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THE HYPERTHERMOPHILIC DIALYSIS LIQUEFACTION UNIT

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1 MATERIALS AND METHODS

1.1 Fermentors

1.1.1 BATCH SET UP

Batch fermentations were conducted in a 2 L foil fermentor (Visual Safety Reactor, VSR, Bioengineering, CH). The reactor was equipped with a pH-control and a redox-measurement. pH was titrated with 2M NaOH. In operation mode the reactor was gassed with 10 L/h-40 L/h CO_2, N_2, or a mixture thereof)

For the preparation of the fermentation1.5 L medium was filled into the reactor and sterilized *in situ* for 20 min at 120 °C. After sterilization the gas phase was replaced by inert gasses and the reactor was heated to fermentation conditions. Non-heat stabile compounds of the medium were added to the reactor by means of a 0.22 µm sterile filter.The liquid phase was reduced with a reduction agent (Cysteine, Na_2S or $Na_2S_2O_3$) and the reactor was inoculated with 50 mL -100 mL pre-culture.

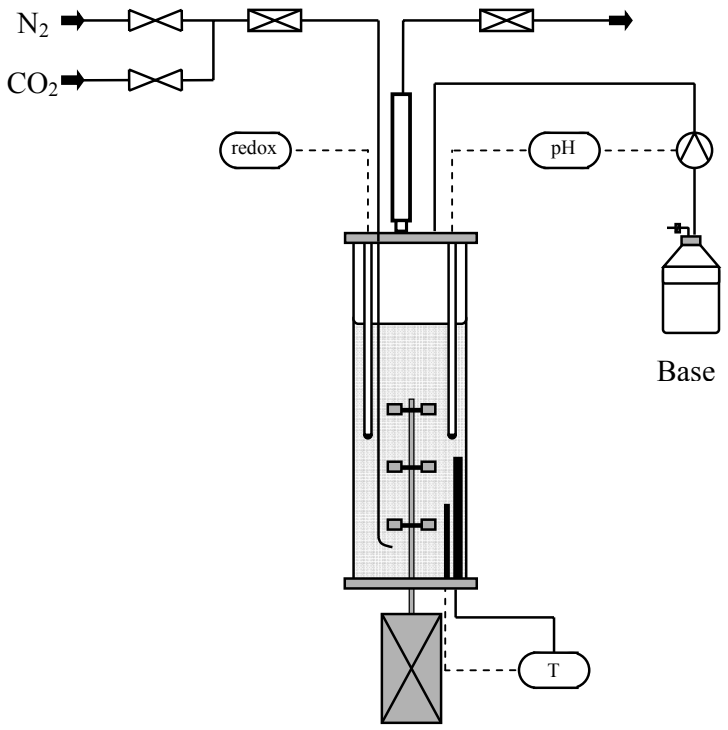

Figure 1: Schematic drawing of the 2L-Bioreactor. Temperature regulation was done with a 800 W Heater. pH was regulated by titration with 2M NaOH; redox-value was measured. If not stated otherwise, the fermentor was gassed with 80% N_2 and 20% CO_2.

1.1.2 CONTINUOUS DIALYSIS MEMBRANE REACTOR

A Bioengineering Visual Safety Membrane Reactor (VSR) was used for dialysis experiments with a total volume of 8 liters. The reaction volume is 1.5 liters, the dialysate volume 4.5 liters. A PES 300kDa UF-membrane [Micro PES-1F/PH 0.1µm Membrana GmbH, Wuppertal, Germany] was used as dialysis membrane. The membrane was glued with a scotch tape [Scotch 3M VHB 9469 19mm x 55m, Firma Bossert, Hamburg, Germany]. pH was maintained at 7.0 by titrating with 2M NaOH. Dialysate was added and withdrawn by peristaltic pumps [Watson-Marlow, 101 U/R]. Effluent and influent pumps [Watson-Marlow, 501U] were triggered by a timer for two minutes every 3 hours. Before pumping the inner stirrer was switched off for one minute. One minute after the pumping ended the stirrer was switched on again. Different dilution rates were achieved by a change in the inlet pump rate. The dialysate chamber was gassed with 10 L/h Nitrogen. Figure 2 shows the fermentor.
ESA-Substrate of 1% and 2% was used as Feed; tap water was used as dialysate. The fermentor was inoculated with 100 mL pre-culture. The fermentation was done unsterile.

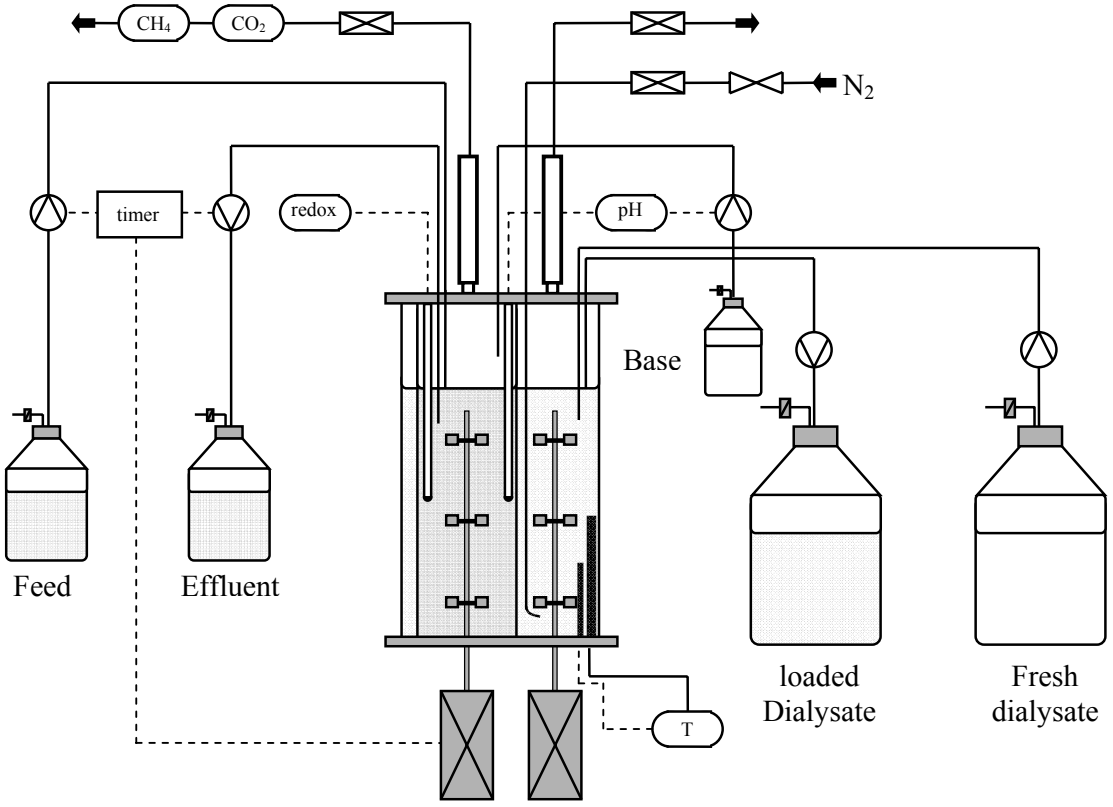

Figure 2: Schematic drawing of the continuous membrane dialysis reactor. The microorganisms and the solid substrate were in the inner chamber; the outer chamber contained the dialysate. The solid matter was dissolved microbially and diffused in dissolved form over the membrane. With the dialysate exchange the dissolved matter is removed from the system. To prevent the input of dissolved oxygen into the system with fresh dialysate, the dialysate was stripped with N_2.

1.1.3 REACTOR WITH SUBMERSED MEMBRANE MODULE

The 6L dialysis membrane reactor was used for further experiments. The fermentor was modified to install a submersed hollow fiber membrane module. Instead of the culture chamber and the dialysate membrane sheet the membrane module was set into the fermentor. Fresh dialysate was pumped through the hollow fibers of the module and was loaded with small molecules.

The bottom part and lid were manufactured from PEEK (poly-ether-ether-ketone). In these plates two holes were made for the connection to the membrane module (Microdyn, MD 070 FP 1N). This module uses PP as membrane material, which has to be hydrophilized with 70% ethanol before usage. The Module was soaked for 24 h in 70% ethanol as recommended by the manufacturer. The hydrophilization lasts, until the membrane dries completely; then it has to be repeated. After hydrophilization, the non adsorbed ethanol is washed away with water.

The hollow fibers are gathered in a matrix made from PU, which is not thermostable. To prevent thermal degradation of the PU cooled dialysate was pumped back onto the top section of the dialysate module to lower the dialysate effluent temperature (not shown).

The fermentor was heated to 90-92 °C by pumping hot water trough the cooling facility of the fermentor. With this set up a stable and continuous heat dissipation was possible. Earlier experiments with an electrical heater lead to boiling water near the heater because of the good insulation characteristics of sedimented solids.

The feed and effluent pump were triggered by a timer and pumped for 2 minutes every 3 hours. Prior to pumping the stirrer was switched off for one minute to allow big particles to settle down. Dialysate was exchanged continuously. When using the micro-filtration membrane two pumps, one at the dialysate inlet and one at the outlet were necessary to prevent a convective volume flow over the porous membrane. The dialysate was stripped with 5 L/h nitrogen and reduced with 0.5 g/L Na_2S x $7H_2O$. Nothing else was added to the dialysate.

pH was titrated by addition of 4M NaOH. Redox potential was measured on line.

The set up is displayed in figure 3.

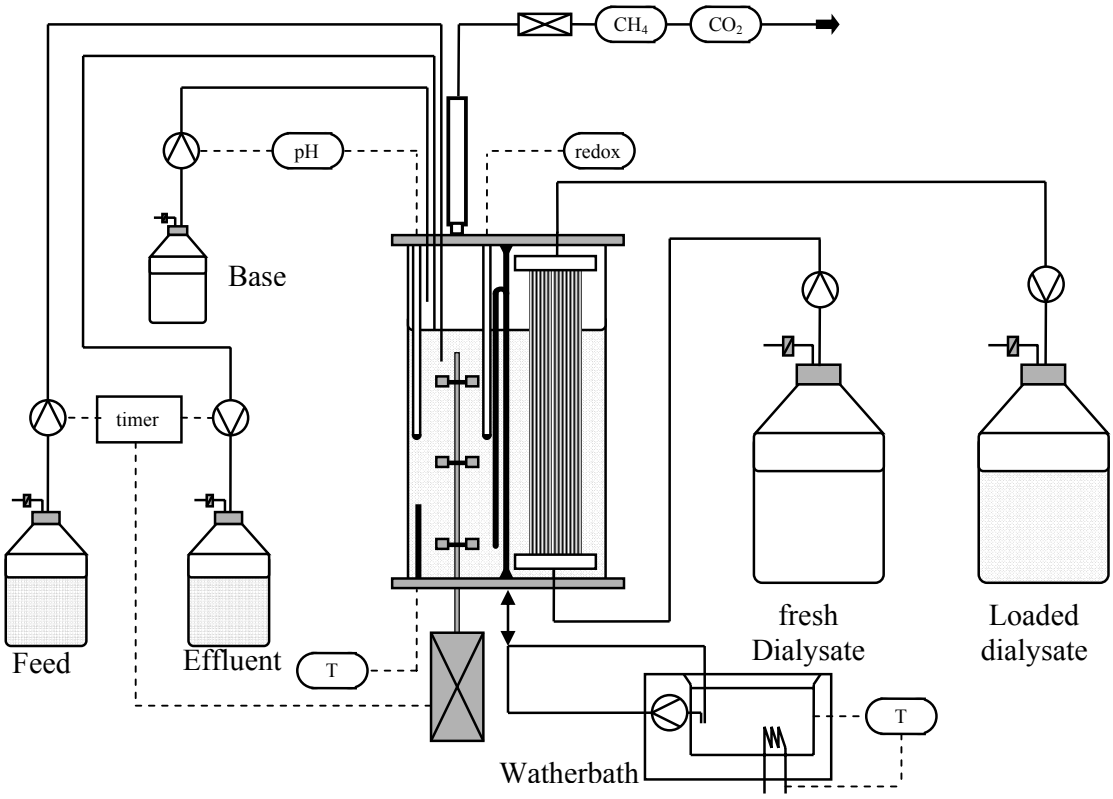

Figure 3: Schematic drawing of the dialysis fermentor with submersed dialysis membrane. The whole reactor was used for cultivation. The dialysis module offers a larger membrane area compared to the dialysis membrane reactor. Dialysate was pumped through the hollow fibers. The reactor was heated by thermostated waterbath which pumped water through the cooling coils of the reactor. To prevent the input of oxygen with the fresh dialysate, the dialysate was gassed with nitrogen and reduced with a reducing agent.

1.1.4 EXTERNAL DIALYSIS MODULE

External dialysis fermentations with *A. thermophilum* (1.2.2) were done with solid free media (1.3.2). For this purpose a 2 L visual safety reactor and a 6 L membrane bioreactor were coupled by means of a dialysis membrane. Fermentor liquid and dialysate liquid were pumped in concurrent mode through the module; fermentor liquid through the hollow fibers, dialysate through the jacket side. The 2 L-reactor was used as described above. The membrane reactor was set up without membrane, but served as 6 L-CSTR. The membranes used in this set up were: Fresenius medical care, Hemoflow highflux HF 80 with a membrane area of 1.8 m², and Fresenius Ultrafilter Typ SPS 7005 – M with a membrane area of 0.1 m². The set up is depicted in figure 4.

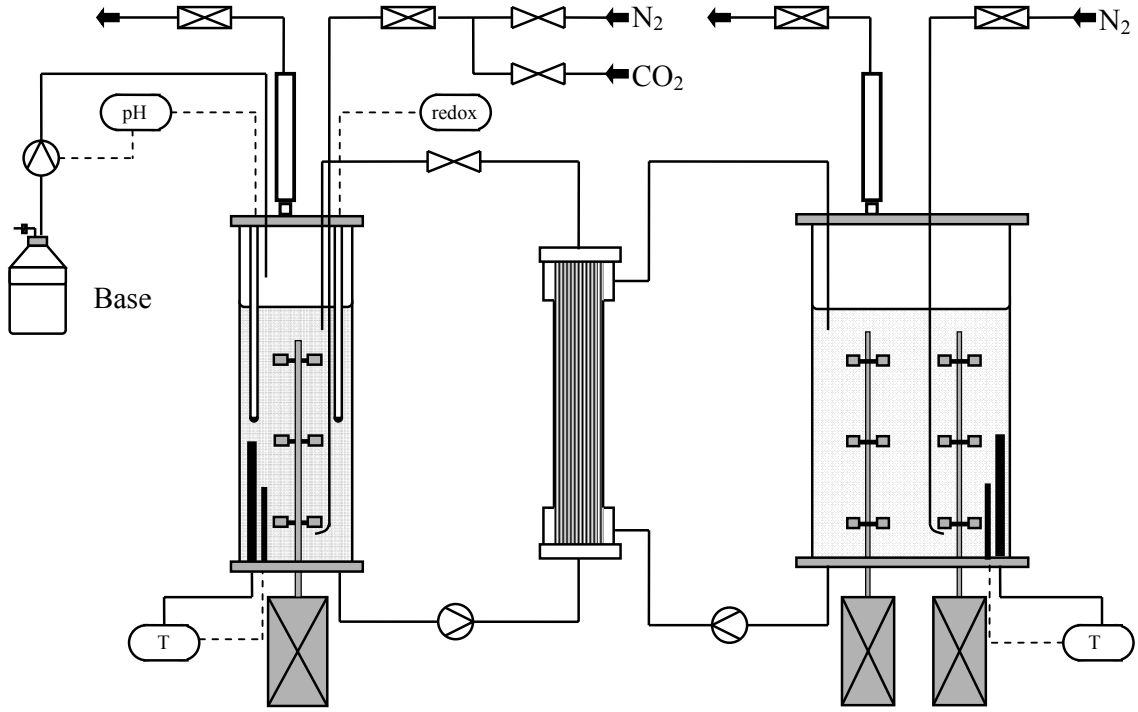

Figure 4: Set up of the fermentor with external dialysis module. The set up was only possible with solid free media. Fermentor broth was pumped through the hollow fibers; dialysate was pumped through the jacket side. The module was used in concurrent operation to minimize pressure gradients over the membrane. The culture chamber was gassed with 80% N_2 and 20% CO_2.

1.1.5 ANAEROBIC RADIAL FIXED BED REACTOR

A 5 L radial flow fermentor was set up for biogas experiments. The fixed bed had a total volume of 3.12 L and was filled with Biolox 10 carriers [Rauschert, a=640 1/m, ε=82%]. The maximal superficial velocity was set to 1 mm/s with a circulation pump. This velocity was commonly used in our lab as the highest possible speed without the release of microorganisms and cell cultures from carriers. However in anaerobic waste water treatment often lower circulation velocities are used (Anderson and Björnson, 2002, Evaluation of straw as a biofilm carrier in the methanogenic stage of two-stage anaerobic digestion of crop residues Bioresource technol 85(1):51-56). The reactor was heated via a heating jacket to 37 °C. Biogas production was determined volumetrically, CO_2 CH_4 and pH was measured online. The biogas fermentor is shown in Figure 5.
The feed vessel was stripped with 10 L/h N_2 to prevent oxygen from entering the system.

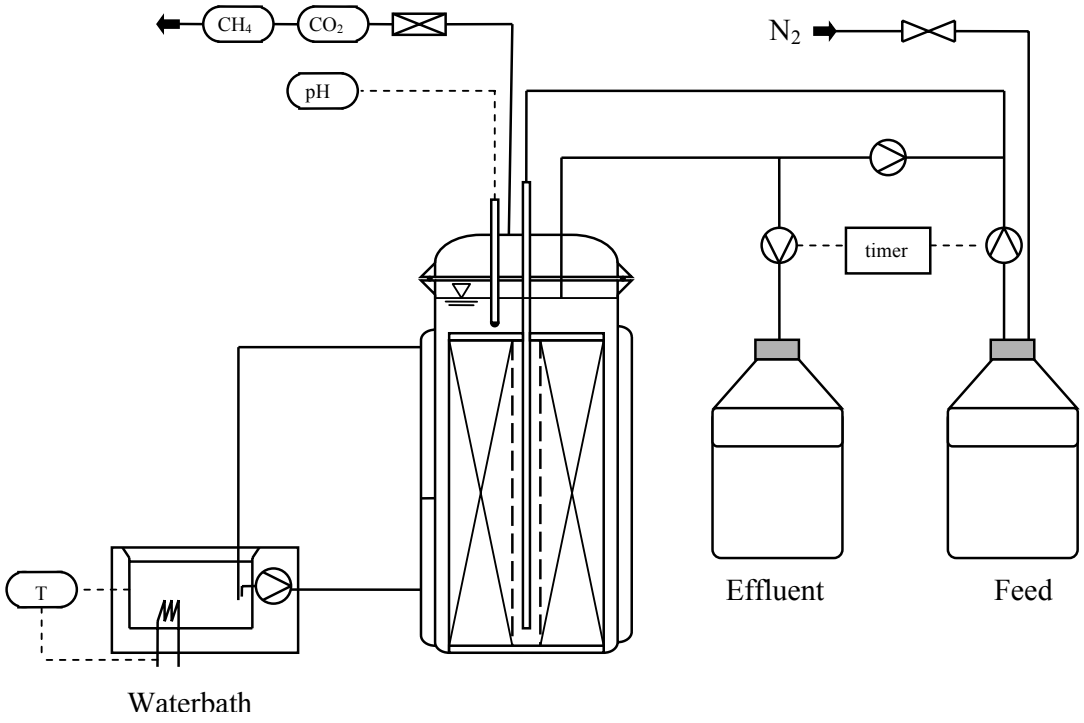

Figure 5: Set up of the radial flow fixed bed reactor for waste-water with low COD loadings. A circulation pump caused a steady flow through the fixed bed by the means of a distribution tube. Addition of feed and withdrawal of effluent was done in the circulation, controlled by a timer. The reactor was heated by a thermostated water bath and a heating jacket.

1.2 Micro organisms

1.2.1 CALDOCELLULOSIRUPTOR LACTOACETICUS

The extremthermophilic Bacterium *C. lactoaceticus* (DSMZ9545) was isolated by Mladenovska et al. (1995) from alkaline hot springs in Iceland. The bacterium is not spore forming. The optimal growing conditions are 70°C and pH7. *C. lactoaceticus* is able to break down cellulose and degrade it to CO2 (8%), acetic acid (18%), lactate (52%), ethanol (1%), hydrogen (1%) and glucose (16%). The conversion was 95% after 3 days.
C. lactoaceticus is also able to degrade pectin and xylose.

1.2.2 ANAEROCELLUM THERMOPHILUM

The extremophilic bacterium *A. thermophilum* (DSMZ6527) was isolated by Svetlichny et al. (1990) from geysers in Kamchatka, Russia. The bacterium is not spore forming. The optimal growing conditions are 75 °C and pH 7.2. *A. thermophilum* degrades Cellulose to lactate and acetic acid, CO_2 and H_2. *A. thermophilum* uses a wide range of carbon sources, such as glucose, mannose, glycogen, fructose, maltose, starch, and sucrose.

1.2.3 CONSORTIUM FROM THE AZORES

In spring 2001 a hyperthermophilic consortium was enriched from different hot sources on the Azores (Portugal). A 2 Liter Visual Safety Reactor (see 1.1.1) was assembled there and run in continuous mode. 2% ESA substrate was used; the hydraulic retention time was 2 d. Fresh medium was prepared with hot water from the following sources: Caldeira grande (T=98 °C, pH 8,4), Caldeira do Asmodeu (T=98 °C, pH 7,4), Caldeira do Esguicho (T=97 °C, pH 8,14), Caldeira dos Inhames (T=95 °C, pH 7,5), Caldeira dos Vimes (T=92 °C, pH 7,4) Agua Santa (T=96 °C, pH 7,9) and three smaller unnamed sources (T=85 °C, pH 7). After 10 days the fermentor had to be titrated with 2 M NaOH to maintain pH 7. A small gas production of 13 mL/(L d) was detected. After 16 days the fermentor was harvested into 100 mL flasks. The cells were stored at 4 °C.

1.2.4 MESOPHILIC ANAEROBIC CONSORTIUM

Non sterile ESA-substrate was inoculated with 1 mL of sewage sludge samples from different wastewater treatment plants and filled into a 1000 mL bottle and stirred for 1 week at 37 °C. The mesophilic consortium showed a broad variety of different single cell organisms. Cocci, rods and spore forming cells have been monitored. No fungi cells or yeasts could be detected optically.

1.2.5 BIOMASS FOR BIOGAS EXPERIMENTS

Biomass was taken from the anaerobic sludge fouling of a local wastewater treatment plant. The biogas reactor was run at 37 °C.

1.3 Media

1.3.1 *CALDOCELLULOSIRUPTOR LACTOACETICUS*

Medium 671 from DSMZ was used. The medium contains the following substances [mg/L]: 1000 NH_4Cl, 100 NaCl, 100 $MgCl_2x6H_2O$, 50 $CaCl_2x6H_2O$, 400 $K_2HPO_4x3H_2O$, 2600 NaHCO2, 750 Yeast extract, 500 Cysteine, 10 mL Trace element solution from DSMZ Medium 141, 1mL vitamin Solution from *Pyrococcus furiosus* medium, 1mL Resazurin solution.
For pre-cultures 2g/L cellulose was used as main carbon source.

1.3.2 *ANAEROCELLUM THERMOPHILUM*

Medium 516 from DSMZ was used. The medium contains the following substances [mg/L]: 330 NH_4Cl, 330 KH_2PO_4, 330 KCl, 330$MgCl_2x6H2O$, 330 $CaCl_2$, 500 Yeast extract, 1500 $NaHCO_3$, 500 Cysteine, 1 mL Resazurin Solution, 10 mL Trace element solution, 1 mL vitamin solution.
For pre-cultures 5 g/L cellobiose was used as main carbon source.

1.3.3 *PYROCOCCUS FURIOSUS,* HYPERTHERMOPHILIC CONSORTIUM

The minimal medium for the hyperthermophilic consortium was adapted from a *Pyrococcus furiosus* medium. The salt content was lowered to 1%. Also the sulphate concentration was decreased. A trace element solution was used described by Belay (1984). The amino-acid cocktail was described by Raven and Sharp (1997). Table 1 shows the minimal medium.

Table 1 composition of the defined medium for the hyperthermophilic consortium. The Medium was adapted by the defined medium for *Pyrococcus furiosus*. (Raven and Sharp, 1992)

Chemicals	[mL/L]
Saltstock	33
Sulfatestock	33
$NaSeO_3$*5H_2O (5mmol)	1
Na_2WO_4*2H_2O (3,3g/l)	1
$FeSO_4$*7H_2O (35g/l)	1
Belay solution	10
Amino acid cocktail	50
Vitaminecocktail	1
Cystein HCl*H_2O	0,5g

Saltstock:	[g/L]
NaCl	300
$CaCl_2$ X 2H_2O	0.66
KH_2PO_4	14
Sulfatestock:	
$(NH_4)_2SO_4$	13
$MgSO_4$ X 7H_2O	2.5

Vitamincocktail	[mg/L]
D-Biotin	20
Cyanobalamin	1
Folic acid	20
Lipoic acid	50
Nicotinic acid	50
DL-Calcium Phantothenate	50
Pyridoxine HCl	100
Riboflavin	50
Thiamine HCl	50

Belay- trace element solution	[g/L]
$CoCl_2$x6H_2O	0.20
$MnCl_2$x4H_2O	0.10
$NiCl_2$x6H_2O	0.10
$ZnCl_2$	0.10
$CaCl_2$x2H_2O	0.05
$CuSO_2$x2H_2O	0.05
Na_2MoO_4x2H_2O	0.05

Amino acid	[mg/L]
L-Alanine	600
L-Arginine HCl	1000
L-Asparagine H_2O	800
L-Aspartate	400
L-Glutamat Na^+	1600
L-Glutamin	400
Glycin	1600
L-Histidin	800
L-Isoleucin	800
L-Leucin	800
L-Lysin HCl	800
L-Methionine	600
L-Phenylalanine	600
L-Prolin	1000
L-Serin	600
L-Threonine	800
L-Tryptophan	600
L-Tyrosine	800
L-Valine	400

1.3.4 ESA-SUBSTRATE

A synthetic substrate was composed, containing different recalcitrant compounds, which can act as bottleneck in the anaerobic degradation of cellulose. The substrate compounds and their concentrations are listed in table 2. Wheat straw contains a high amount of lignin, algae (*Spirulina platensis*) contain large quantities of protein; soy pellets are composed mainly from both protein and lignocellulose. A detailed description is given in table 3. The original compounds feces and cabbage were omitted from the beginning, because they could not be ground to a particle size smaller than 1mm and therefore plugged the piping system in the continuous reactors. However these parts of the ESA-substrate were not the bottlenecks of a

fast degradation at 90 °C. The biosafety-problems with the handling of feces were non-existent because of the high working temperature.

Table 2: Composition of the ESA-substrate, dry matter content, COD, Total carbon and nitrogen

	Mass-%	Dry matter content	COD of dry matter	TC of dry matter	TN of dry matter
	[g_{DM}/g_{DM}]	[g_{DM}/g]	[g_{COD}/g_{DM}]	[g_C/g_{DM}]	[g_N/g_{DM}]
straw	0.41	0.93	1.3	0.39	0.0087
soy	0.41	0.88	1.21	0.39	0.0166
algae	0.18	0.95	1.49	0.42	0.1027

Table 3: More detailed composition of the dry ESA substrate. Concentrations of fat, protein, fibrous matter, its composition and Ash content are given. Data from manufacturer (BlueBiotech, Elmshorn, Germany, algae), from Landwirschaftskammer-NRW (www.landwirtschaftskammer.de, soy) and from Alfani et al. (2000) (wheat staw)

	Algae	Soy	Wheat straw	total
Fat	5%	2%	7%	5%
Protein	64%	49%	3%	32%
Starch		6%		2%
other carbohydrates	13%	11%		7%
fibrous matter	9%	8%	82%	38%
-Cellulose		4%	34%	16%
-Hemicellulose		2%	26%	11%
-Lignin		2%	22%	10%
Ash	8%	8%	8%	8%

1.4 Methods

1.4.1 DOC

The DOC and TIC value of the samples was determined with TOC + TN_b from Analytic Jena. Part of the sample is burnt at 800°C in a pure oxygen atmosphere in a column filled with carriers of Cer catalyst. The CO_2 content in the off-gas is integrated and the total carbon (TC) determined. The Total Inorganic Carbon (TIC) is determined by acidifying the sample to pH 2 and flushing out the dissolved CO_2

1.4.2 VFA

VFA are determined with a headspace gas chromatograph Chrompack CP9001. A 30mx0.32mm Nukol capillary from Supelco is used. Carrier gas is nitrogen. The samples are acidified with 2% H_3PO_4. The column is heated to 60°C, after tree minutes the temperature rises with 10°C/min to 200°C. The detector is a FID working at 220°C. The following VFA can be measured: C2, C3, i-C4, n-C4, i-C5, n-C5, n-C6, n-C7

1.4.3 AMINO ACIDS

Amino acids are determined with HPLC analysis. The samples are derivated with OPA agent containing phthaldialdehyde, 2-mercaptoethanol and methanol in Borate buffer at pH 9.5. The detector was a fluorescence detector.

1.4.4 PROTEINS (LOWRY-ASSAY)

The Lowry assay uses the ability of proteins to form colored complexes with copper under alkaline conditions. A copper agent is prepared form the following solutions. 0.5mL K-Na-Tartrate (4% w/v), 0.5 mL $CuSO_4$ (2% w/v) and 99 mL Na_2CO_3 (3% w/v) are mixed and stored at 4 °C for a maximum of two weeks. Folin-Ciocalteau agent is mixed with 50% demineralized water.
100 µL Sample is added to 1 mL copper agent and incubated at room temperature for 15 minutes. 100 µL Folin agent are added afterwards. The mixture is incubated for another 30 minutes. The adsorption at 660 nm is measured against a blind sample.
The protein content is calculated with the help of a calibration curve of 0.02 g/L-0.4 g/L bovine serum albumin (BSA). All measurements are done in duplicate.

1.4.5 CARBOHYDRATES

Carbohydrates are determined by measuring the reducing sugars after acidic hydrolysis of the sample. Anthron solution is prepared from 200 mg Anthron agent, dissolved in 5 mL ethanol and filled up to 100 mL with 75% Sulphuric acid.
0.5 mL Sample and 2 mL Anthron Solution are given into a cuvette and are incubated for 10 min at 100 °C. The reaction is stopped on ice. The absorption at 635nm is measured against demineralized water. The carbohydrate content is calculated with a calibration curve made with 1% autoclaved starch solution. The test is valid between 5 and 100 mg/L. Samples with higher carbohydrate concentration are diluted with demineralized water.
reduced sugars
5g 2-Hydroxy-3,5-Dinitrobenzoic acid are dissolved in 100ml 2M NaOH. 250g Rochelle's salt are dissolved in 250ml demineralized water. Both solutions are poured together.
250µL of the DNS-solution are added to 500µL 0.05M Sodiumacetate buffer pH4.7. 250µL Sample is added. The mixture is incubated at 100°C for 5 minutes and cooled down on ice. The extinction is determined at 540nm. A calibration curve, done with glucose can be used to calculate the amount of reduced sugars. The test is valid from 0.05 to 3g/L glucose.

1.4.6 GC-MS

Gas chromatograph mass spectrometer analysis was used for qualitative non target screening.

Samples were extracted 1:1 in dichloromethane. The detector was a Hewlett Packard. Model HP 5971 A with GC-5890 Series II.

1.4.7 TOTAL SOLIDS

Two samples of 10 mL each are filled in previously dried and weighed ceramic beakers. The samples are weighed again. Then the beakers are dried at 105 °C for 48 hours. After this the beakers are cooled down and weighed again. The TS-value is calculated by dividing the mass difference after drying by the mass difference before drying. The arithmetic average of the two measurements is determined

2 CULTIVATION OF THERMOPHILIC ANAEROBIC MICROORGANISMS CAPABLE OF DEGRADING LIGNOCELLULOSE IN DEFINED MEDIA

2.1 Anaerocellum thermophilum

A. thermophilum was grown in a 2-Liter reactor at 70°C, using cellobiose and yeast extract as sole carbon source. pH7. Figure 6 shows the cell density and optical density, the use of substrate and the formation of the metabolites lactate and acetic acid. The fermentation showed a depletion of cellobiose after 15 h. After this, both cell density and optical density showed a minor decline, but remained constant for another 25 h.
Unlike many thermophiles, *A. thermophilum* displayed no strong lysis phase. From the cultivation of other anaerobic bacteria and archaea, it is known, that stirring and gassing has a strong influence on the growth and product formation. Gassed cultures of *A. thermophilum* showed slower growth than ungassed cultures, regardless of the used gasses (CO_2, N_2, and mixtures thereof, data not shown). However the variation of stirrer speed resulted in an interesting observation. The fermentation at 500 rpm, 750 rpm, and 1500 rpm are shown in figure 7.

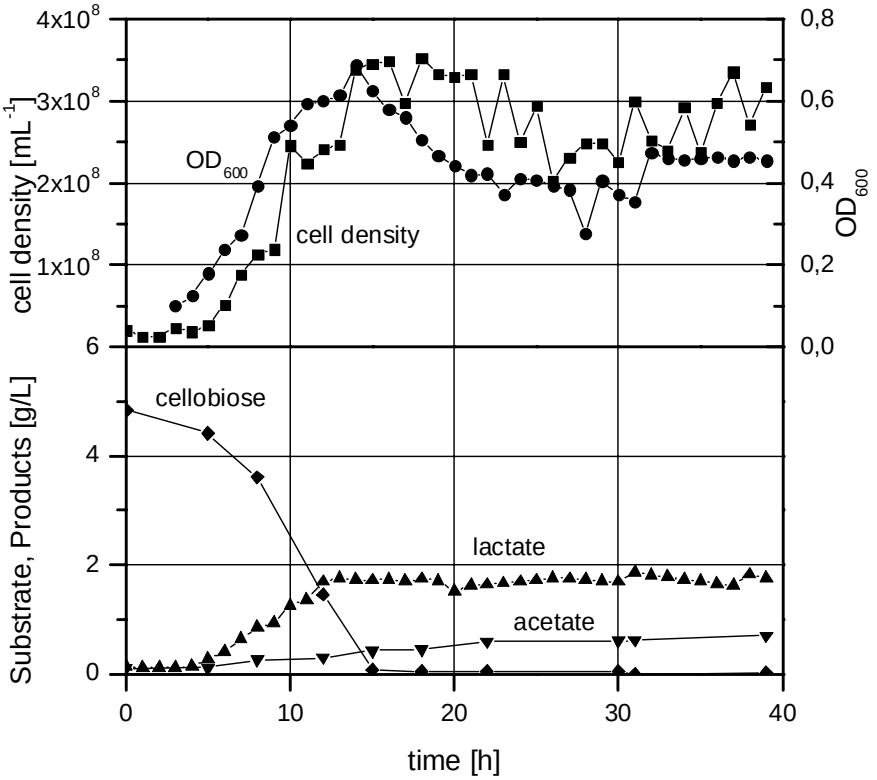

Figure 6: Growth of *A. thermophilum* at 70 °C and pH 7 in a 2 L reactor filled with mineral medium supplemented with 5 g/L cellobiose and 0.5 g/L yeast extract. The reactor was not gassed and was stirred with 500 rpm. The substrate was used up completely till 15 h and transformed to lactate and acetate.

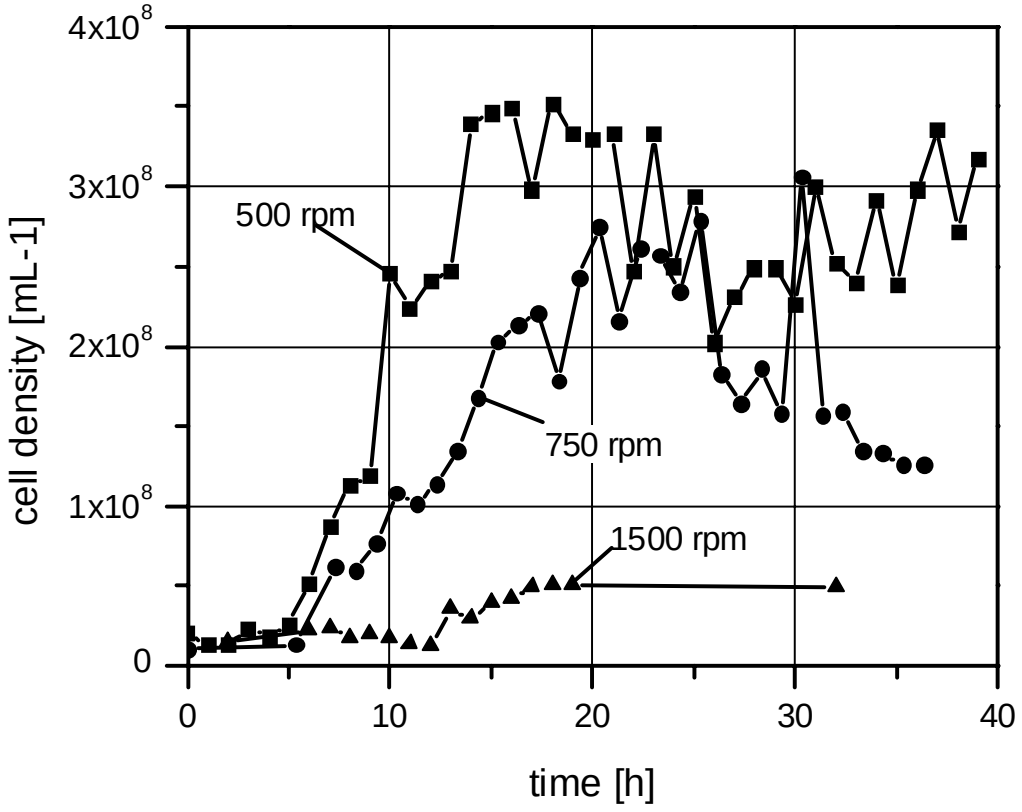

Figure 7: Growth of *A. thermophilum* in mineral medium with yeast extract and cellobiose at 70 °C and pH 7. Stirrer speeds of 500 rpm, 750 rpm and 1500 rpm were applied. At high stirrer speeds the growth declined but the substrate was still used up completely.

With increasing stirrer speed, the growth slowed down, but even at high stirrer speed and low biomass yields the substrate cellobiose was transformed completely. A more detailed view of this behavior is given in figure 8. Here the carbon balance of the liquid phase is shown. The time used for complete utilization increased with increasing stirrer speed. Also a shift of metabolic end products can be seen. At 500 rpm, approximately 33% of the ingoing carbon was transformed to lactate. At 750 rpm the lactate fraction was 45%, and at 1500 rpm, lactate covered 66% of the carbon balance. Mladenovska et al. (1994) describes the formation of lactate, acetate, hydrogen, and ethanol as main metabolic end products for *A. thermophilum* and *C. lactoaceticus*.

A possible explanation for the accumulation of lactate at high stirrer speed is the better mass transport of hydrogen form the liquid to the gaseous phase. Without gassing and at low stirrer speed hydrogen accumulated in the liquid phase. $NADH_2$ is produced during the glycolysis. It cannot be regenerated to NAD in the presence of high hydrogen concentration in the liquid phase (Ruzicka 1996). Another way to regenerate ethanol is the formation as ethanol from pyruvate via acetaldehyde. Instead of hydrogen, ethanol acts as a final electron acceptor.

A similar effect was observed during the fermentation of *P. furiosus* at elevated hydrogen concentrations (Krahe 1998). At higher partial pressures of hydrogen, more reduced metabolites accumulated. Instead of ethanol, which is produced by *A. thermophilum* and *C. lactoaceticus*, *P. furiosus* produces alanin.

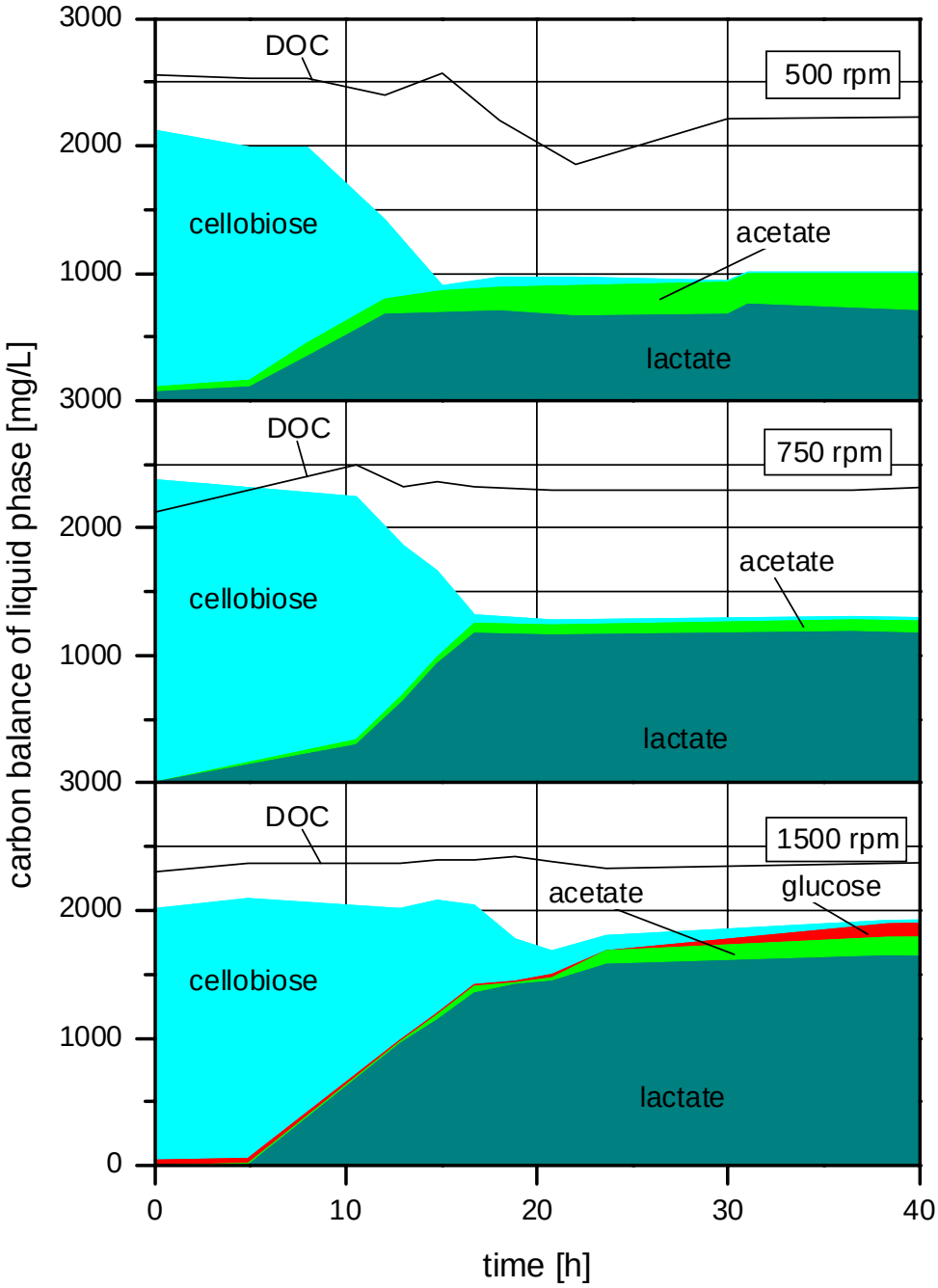

Figure 8: Carbon balance of the liquid phase at the ungassed batch cultivation of *A. thermophilum* at 70 °C and pH 7. The stirrer was set to 500 rpm, 750 rpm and 1500 rpm.
5 g/L cellobiose and 0.5 g/L yeast extract were used as substrate. Throughout the experiment the substrate was converted to lactate, acetate, glucose and an unknown metabolic end product (probably ethanol). The amount of

dissolve organic carbon did not change significantly over time. With higher stirrer speeds, the amount of lactate increased, while the amount of the unknown metabolite decreased.

A. thermophilum displayed a good stability towards mechanical stress in the absence of substrate at low stirrer speeds. At higher stirrer speeds, the growth and maximal cell density decreased. Therefore the next experiments were all carried out at 500 rpm. For the increase of biomass concentrations dialysis fermentations were done. Two different set ups were used and compared to the batch experiment at 500 rpm. The experiments are shown in figure 9. At first the dialysis membrane reactor was used. The reactor was filled with medium; substrate was added on both sides of the membrane. After a lag phase of 8 h, *A. thermophilum* started its growth and reached $6.5x10^8$ cells per milliliter at the end of exponential phase which could be observed after 24 h. At this point the substrate cellobiose was used up completely. In the stationary phase the cell density showed some fluctuations, but in general stayed constant till the end of the experiment after 42 h.

Since the dialysis membrane reactor cannot be scaled up, a second set up with an external dialysis module was used. In this set up, fermentor liquid is pumped from the fermentor to hollow fiber module, where the mass transport takes place, and then back again into the fermentor. A lag phase of 4 h preceded the exponential growth phase, which came to an end after 27 h, reaching 5.5×10^8 cells per milliliter. The plot of the substrate showed a plugging of the membrane starting between 15 h and 25 h. The substrate concentration in the cultivation chamber reached zero after 25 h, but the dialysate chamber still contained 2 g/L cellobiose. This indicates the plugging of the membrane and is visualized in figure 10. After the fermentation the module was tested and the plugging confirmed. The membrane was regenerated by flushing with hot citric acid, acting as a complexing agent. Probably the high calcium concentration and the increase of pH due to the withdrawal of the acids in the module were responsible for the formation of precipitates, which covered the membrane and therefore prohibited the diffusion of small molecules over the membrane. The solubility products are $L(CaCO_3)$=0,5-1 x 10^{-8} mol^2/L^2, $L(Mg(OH)_2)$=0.6-1 x 10^{-11} mol^3/L^3.

Both dialysis experiments showed a better growth of *A. thermophilum* than the batch experiment, which reached 3.5×10^8 cells per milliliter.

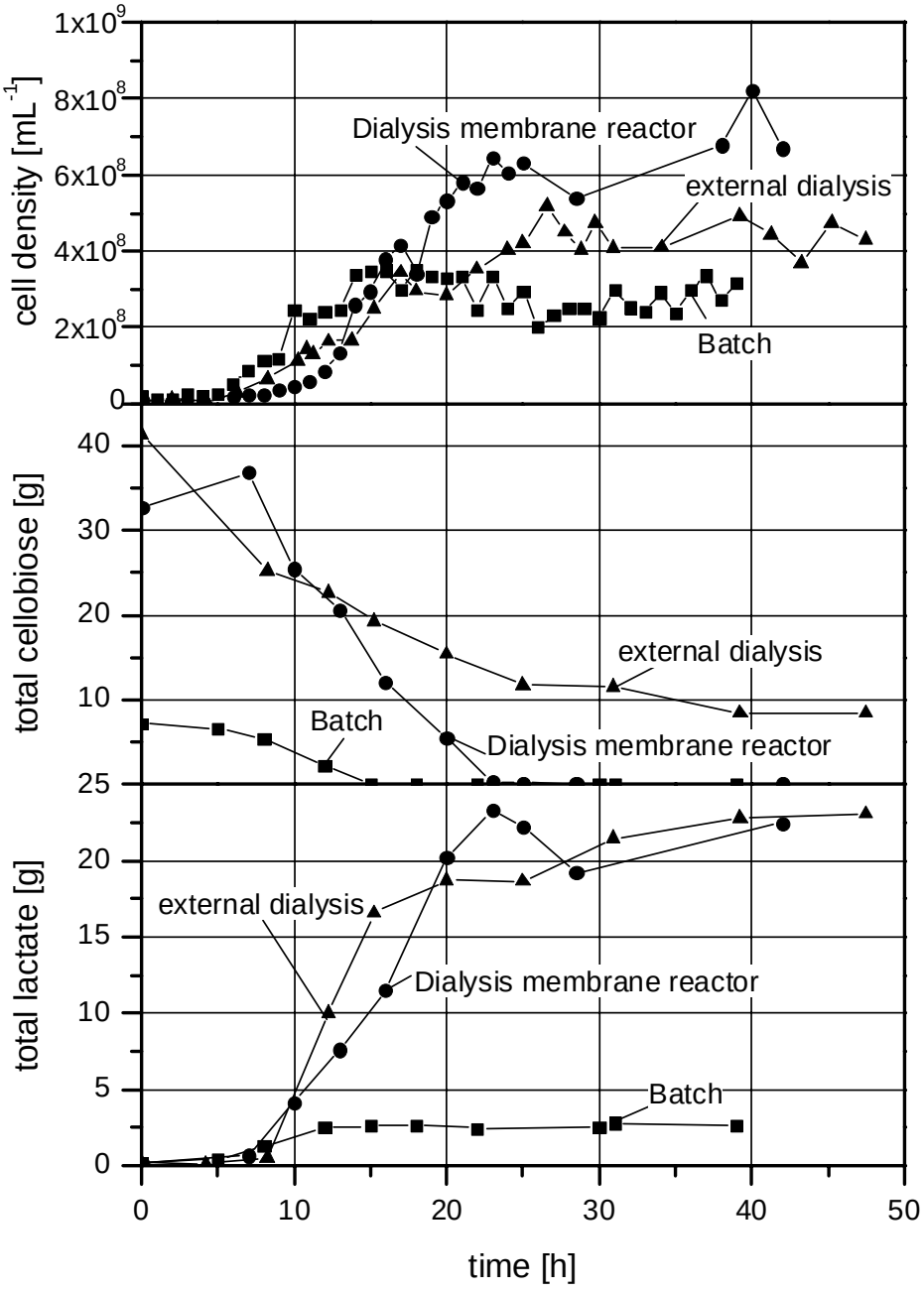

Figure 9: Growth of *A. thermophilum* at 70 °C, pH 7 in a mineral medium with cellobiose and yeast extract. Three types of reactors were used: batch reactor, dialysis membrane reactor and reactor with external dialysis module. Plotted are the cell density, the substrate cellobiose and the main metabolite lactate.

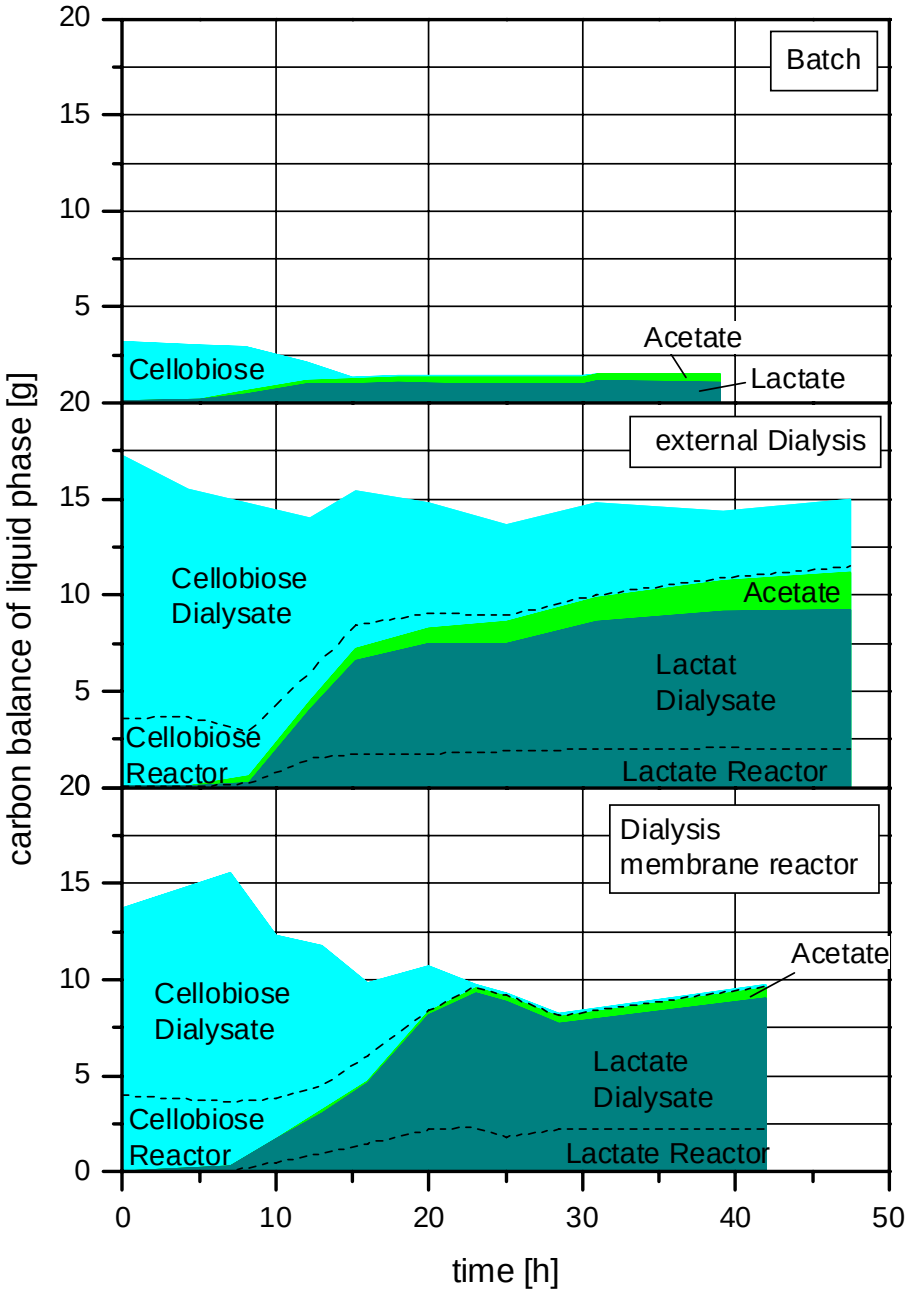

Figure 10: Carbon balance of the batch and dialysis fermentations of *A. thermophilum* at 70 °C, pH 7 in a mineral medium with cellobiose and yeast extract. During fermentations in batch and dialysis membrane reactor the substrate was used completely, while using the external dialysis module, the module plugged at 22 h and no substrate could cross the membrane.

2.2 *Caldocellulosiruptor lactoaceticus*

C. lactoaceticus was cultivated in a 2-L bioreactor with no gassing and 500rpm stirrer speed. Other cultivation conditions were: pH 7, T=70 °C. 2 g/L Cellulose and 0.5 g/L yeast extract were used as carbon source. Unlike cellobiose, cellulose is a non soluble carbon source. Before it is taken up by the bacterium, it has to be degraded by a mixture of cellulases, secreted by *C. lactoaceticus*. In the suspension of cellulose particles the optical density could not be used to monitor bacterial growth. Also the accuracy of the cell count suffered from the presence of cellulose particles of the same size as the bacteria. The growth curve is displayed in figure 11 together with the formation of the metabolites acetate and lactate. After a lag phase of 6 h, exponential growth started with a growth rate of μ=0.23 h^{-1}. The retardiation phase began approximately at 20 h and cell density began to fluctuate and showed only a slight increase.

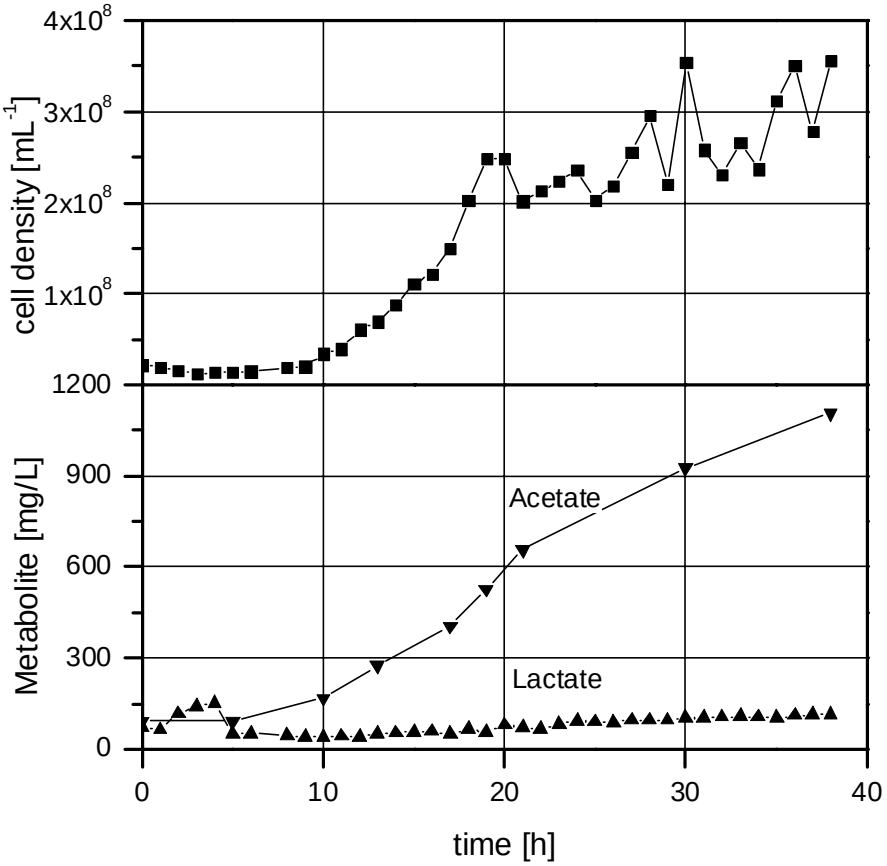

Figure 11: Batch cultivation of *C: lactoaceticus* in a mineral medium supplemented with 2 g/L cellulose and 0.5 g/L yeast extract. Fermentation conditions were 70 °C, pH 7, no gassing, and 500 rpm. Plotted are the growth of the bacteria and the formation of metabolites, mainly acetate.

Dissimilar to *A. thermophilum*, *C. lactoaceticus* produced mainly acetate as metabolic end product. Its production continued throughout the complete experiment. A concentration of 1.1 g/L was reached after 38h. This corresponds to a 40% conversion on a carbon base.
Fermentation of *C. lactoaceticus* at higher stirrer speeds lead to similar results compared with *A. thermophilum*. Again, an increasing stirrer speed slowed down the growth. The presence of cellulose particles caused a high counting error, so the cell density plots are not showed. However, the metabolites could be measured and are displayed in figure12. This figure shows the carbon balance of the liquid phase. At the beginning of the experiments just a small part of the carbon was accessible as dissolved organic carbon (approximately 200 mg_C/L). In all fermentations similar concentrations of DOC and acetic acid were achieved, but the fermentation time increase significantly from 38 h at 500 rpm over 103 h at 750 rpm to 250 h at 3000 rpm.
These observations are in good agreement with Gunijkar et al. (2001), who measured a decrease of activity for cellulases at high stirrer speeds. The cellulolytic activity is obviously the limiting factor, since no intermediate products like, glucose or cellobiose could be found in high concentrations.

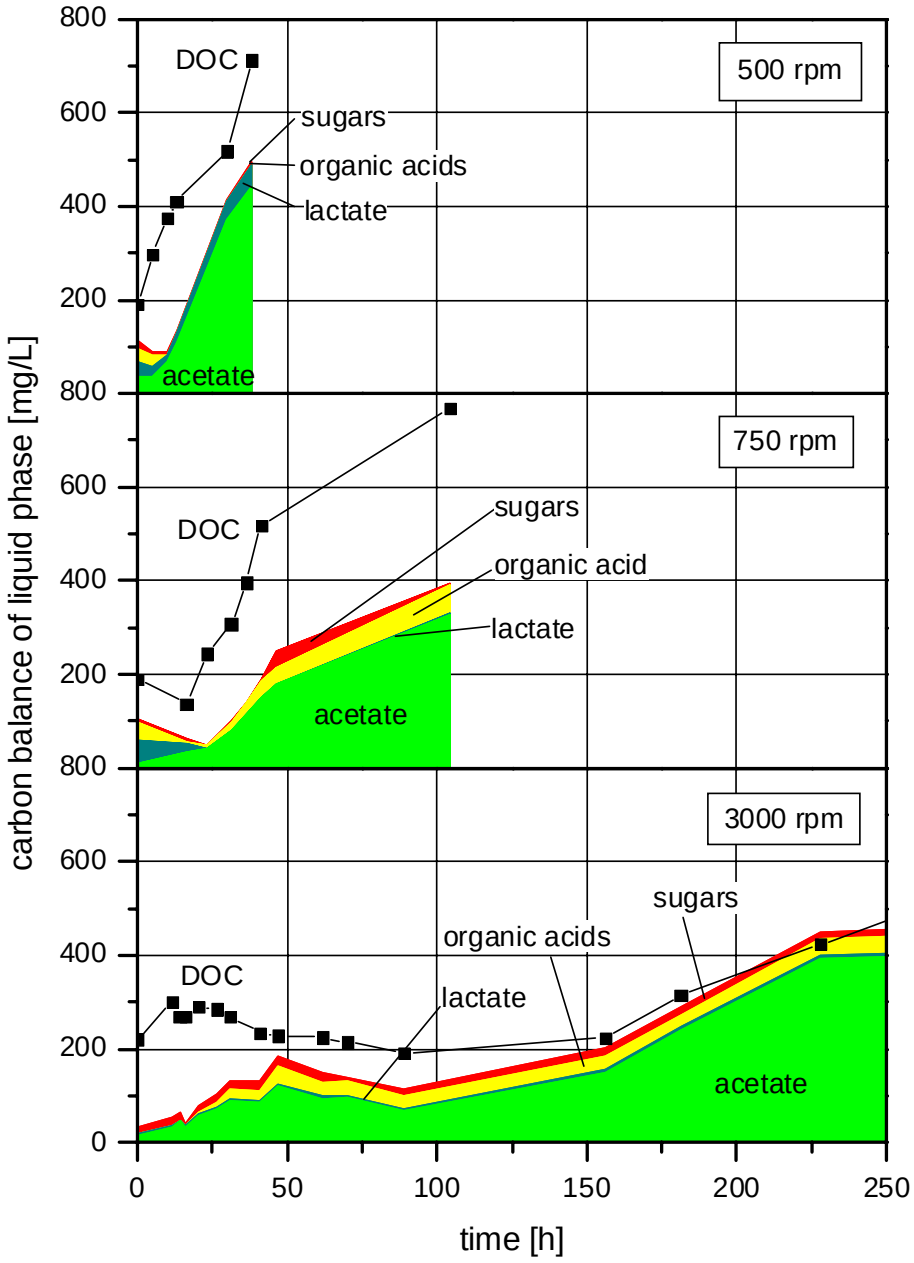

Figure 12: carbon balance of the liquid phase in batch cultivations of *C. lactoaceticus* at 70 °C, pH 7 in a defined medium with 2 g/L cellulose and 0.5 g/L yeast extract at different stirrer speeds. Plotted are acetate, lactate, VFA (C3-C7), soluble sugars (measured as cellobiose-equivalents), and DOC.

2.3 hyperthermophilic consortium

The hyperthermophilic consortium was grown in a 2 L bioreactor at 90 °C and pH 7 in a defined medium (1.3.3) using 10 g/L starch as sole carbon source. The nitrogen demand was covered be an amino acid cocktail comprising of all 20 amino acids except cysteine , its composition published by Raven and Sharp (1997) for the fermentation of the anaerobic hyperthermophile *P. furiosus*. A similar behavior with respect to agitation compared with *C. lactoaceticus* and *A. thermophilum* was detected. At 500 rpm a better growth than at 1000 rpm was detected. Unlike the extremthermophilic bacteria, but comparable to *P. furious* a strong lysis phase becomes evident at the end of the growth phase. No stationary phase was observed.

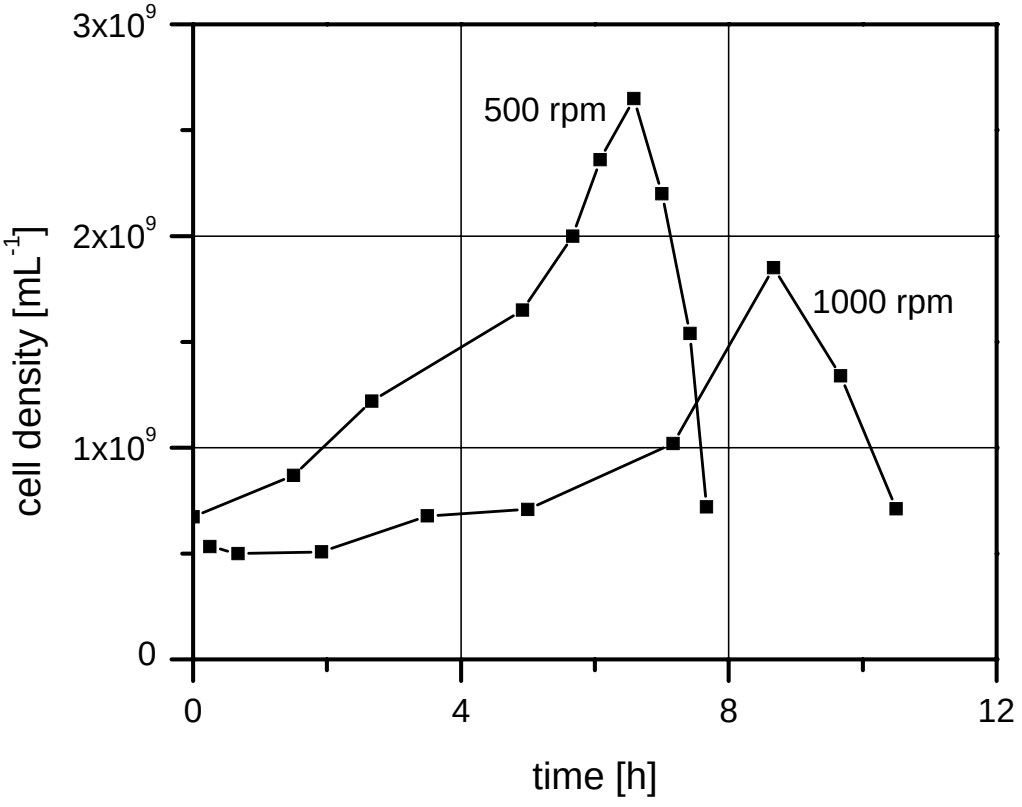

Figure 13: Growth of the hyperthermophilic consortium in a defined medium with 10 g/L soluble starch and an amino acid cocktail at two different stirrer speeds. The experimental conditions were: pH 7, 90 °C, gassing with 30 L/h N_2, 10 L/h CO_2. Similar to *P. furiosus* the hyperthermophilic consortium showed a strong lysis at the end of the fermentation.

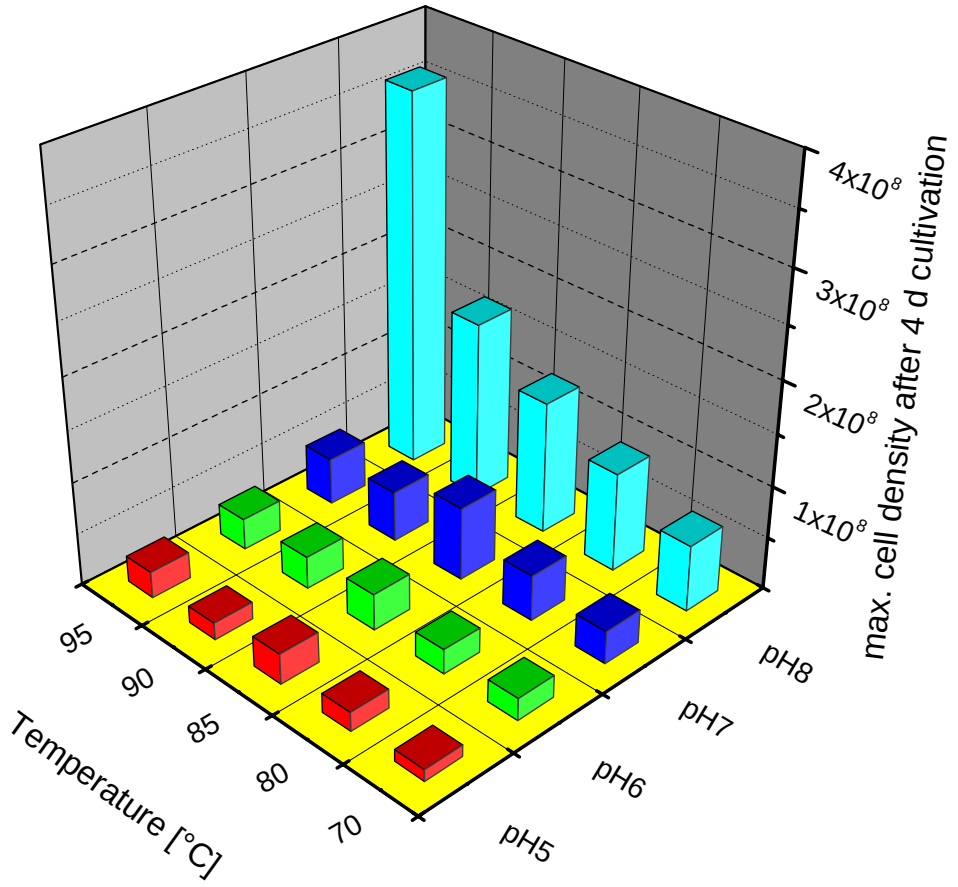

Figure 14: growth of the hyperthermophilic consortium in a pH range from 5-9 and a temperature range from 70 °C-95 °C in 50 mL-vials filled with sterile supernatant of the ESA-medium. At pH5, 6, and 7 the optimal temperature was 85°C, whereas at pH8 the maximum growth was at 95°C. This shift was probably due to different strains in the consortium growing at different optimal conditions.

A screening for optimal growth conditions on the sterile supernatant of autoclaved ESA-substrate revealed a maximal growth at pH8 and 95°C. The screening is shown in figure 14. Additional experiments at pH8 and 100 °C yielded a maximal cell density of 9 x 10^8 1/mL. However, these results do not implicate the best liquefaction performance under this condition, since the screening media contained no solid particles. Instead a non cellulolytic microorganism could have been growing here.

Identification of the consortium with 16S rDNA DGGE failed; see TN 3.7 for further details.

3 DEGRADATION OF LIGNOCELLULOSIC SOLIDS UNDER THERMOPHILIC ANAEROBIC CONDITIONS

3.1 Pre-experiments

Lignocellulose is often regarded as inert to anaerobic degradation (Kirk and Farrel, 1987; Scherer et al., 2000). Before the degradation of lignocellulose containing solids with extremthermophilic and hyperthermophilic microorganisms is examined in detail, the impact of operating conditions (i.e. high temperature) needs to be assessed. ESA-substrate at concentrations of 1% and 2% respectively was therefore incubated at temperatures between 20 °C and 100 °C for one hour. Remaining solids as well as dissolved organic carbon were measured, allowing to determine the extent of spontaneous solubilisation.. It is displayed in figure 15. At ambient temperature the solubilization was found to be 22%, whereas at 100 °C the solubilization was 30%. Both total solids and dissolved organic carbon measurements yielded the same solubilization. However, the results from the total solids measurement displayed a greater experimental error. The solubilization was the same at 1% and 2%.
As a result, all further solid matter degradations are calculated on the basis of the undissolved solid matter at the working temperature. For the solid matter degradation at 90 °C this means, a solid matter content in the feed of 70% is assumed, whereas a solid matter content of 78% was measured in the feed, not heated to 90 °C prior entering the reactor.

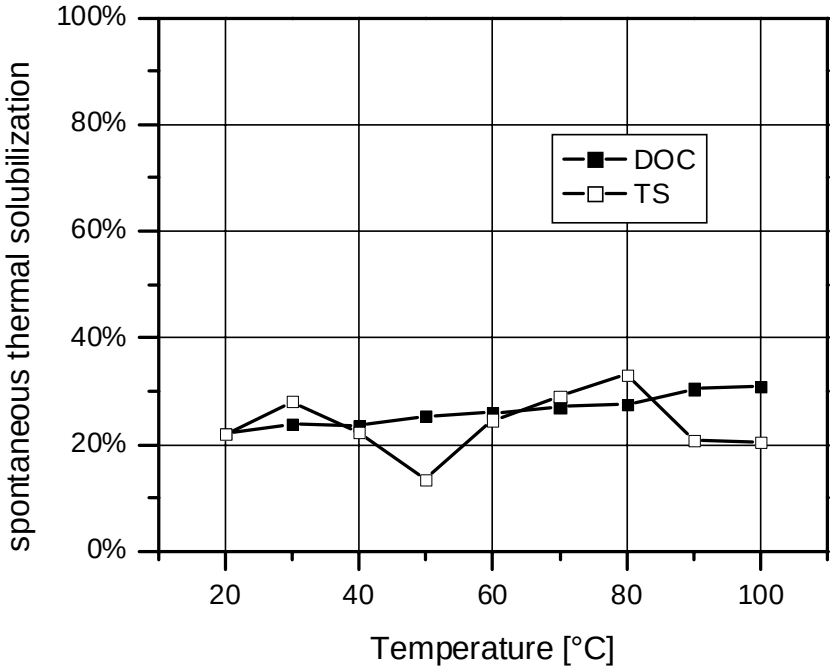

Figure 15: Thermal solubilization of the ESA-substrate depending on the temperature. The substrate was incubated without microorganisms for 1 h at temperatures between 20 °C and 100 °C.

The balances of the continuous dialysis fermentations were calculated on the assumption, that no solid matter accumulates in the fermentor, or that the accumulation can be neglected. Figure 16 proves this assumption. It shows the concentrations of solids in the feed, effluent and fermentor during a continuous 26 d dialysis experiment at mesophilic conditions. The exact experimental conditions were 37 °C, 1% Feed, volumetric loading rate of B_V=2 g/(L d), hydraulic retention time in the culture chamber 7.5 d, hydraulic retention time in the dialysate chamber 0.5 d.

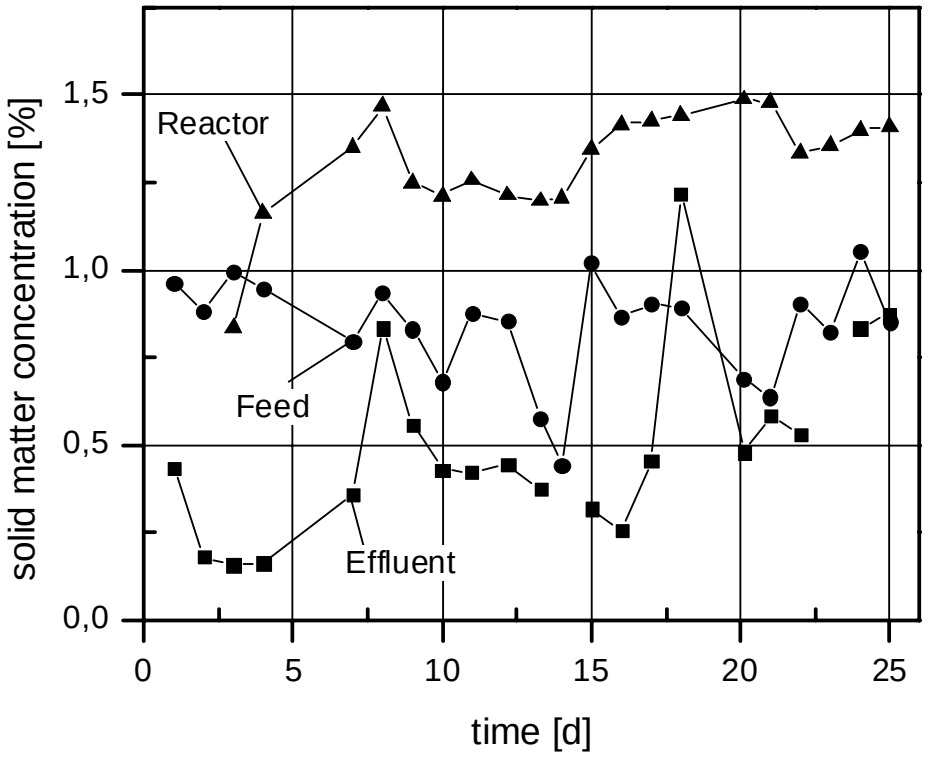

Figure 16: Solid matter concentration in the feed, the reactor, and the effluent during a continuous dialysis experiment (37°C, pH7, B_V=2 g/(L d)). The solid matter content of the reactor increased from 1% to maximal 1.5%. No accumulation was found.

Further experiments were conducted with a broad variation of temperature (37 °C – 100 °C), volumetric loading rate (2 g/(L d) – 13 g/(L d)), Feed concentration (1% and 2 %), pH (pH 7 and pH 8), hydraulic retention times of reactor (1 d – 12 d) and dialysis (0.25 d – 1 d) and reactor types (dialysis membrane reactor and submersed dialysis module). A total number of 10 experiments are shown in the following tables. Table 3 contains all the measured data, table 4 contains the calculated results.

Experiments 1-3 and 10 were all performed at the same loading rate of 2 g/(L d) at different temperatures of 37 °C, 70 °C, 90 °C, and 100 °C respectively. pH of experiment 10 was also different (1 unit). The rest of the experiments were all carried out at 90 °C. Experiment 4 and 5 were conducted with higher feeding rates; in experiment 6 also a higher feed concentration

(2%) was used. During the next three experiments (7-9) a different membrane set up was used: the submerged hollow fiber module.
Experiment 7 ad experiment 8 were carried out under almost exact same conditions, apart from the pH which was shifted from pH 7 in experiment 7 to pH 8 in experiment 8. In experiment 9 a three times greater pumping rate in the dialysate circuit was used, compared to experiment 8.
During the last experiment 10 the temperature was set to almost boiling temperature (99.5 °C); here again the dialysis membrane reactor was used.

Table 3 continuous dialysis experiments - measurements.

Experiment		1	2	3	4	5	6	7	8	9	10
Temperature	[°C]	37	70	90	90	90	90	90	90	90	99.5
pH	[-]	7	7	7	7	7	7	7	8	8	8
Feed-concentration	[-]	1.0%	1.4%	1.4%	1.0%	1.0%	2.0%	2.0%	2.0%	2.0%	2.0%
V. Reactor	[L]	1.5	1.5	1.5	1.5	1.5	1.5	5	5	5	1.5
V. Dialysate	[L]	4.5	4.5	4.5	4.5	4.5	4.5	--	--	--	4.5
Pump. Dialysate	[L/d]	--	--	--	--	--	--	20	20	60	--
Membrane		DMR	DMR	DMR	DMR	DMR	DMR	Module	Module	Module	DMR
Volume-flows											
Feed	[mL/d]	300	300	300	702	1115	983	1221	1241	1356	153
Effluent	[mL/d]	198	185	126	1058	987	1295	894	857	1236	142
Dialysate in	[mL/d]	8118	9000	9000	4500	4990	4500	18861	18767	20837	18335
Dialysate out	[mL/d]	8220	9115	9174	4224	5118	4188	19046	20865	23430	18425
Concentrations											
Solids. Feed	[g_C/L]	3.047	4.000	4.000	2.819	2.819	5.638	5.638	5.638	5.638	5.556
DOC. Feed	[g_C/L]	0.953	1.508	1.676	1.181	1.181	2.362	2.362	2.362	2.362	2.444
Solids. Effluent	[g_C/L]	1.689	2.103	2.372	1.256	1.739	2.562	5.810	5.656	4.234	1.213
DOC. Effluent	[g_C/L]	0.272	0.368	0.294	0.369	0.650	0.685	1.818	1.644	1.344	0.292
HAC. Effluent	[g_C/L]	0.014	0.019	0.015	0.035	0.050	0.064	0.089	0.097	0.104	0.002
VFA. Effluent	[g_C/L]	0.015	0.021	0.015	0.018	0.023	0.024	0.085	0.088	0.038	0.001
DOC. Dialysate	[g_C/L]	0.030	0.088	0.104	0.165	0.272	0.531	0.105	0.116	0.095	0.034
HAC. Dialysate	[g_C/L]	0.008	0.015	0.012	0.022	0.040	0.027	0.015	0.019	0.010	0.002
VFA. Dialysate	[g_C/L]	0.010	0.003	0.009	0.011	0.012	0.011	0.005	0.009	0.001	0.001

DMR = Membrane reactor with 0.05m² PS-Membrane. Module = Micro filtration module PP. 1.1m² Membrane area

DOC = dissolved organic carbon. VFA = volatile fatty acids (C3, C4, i-C4, C5, i-C5, C6, i-C6, C7). HAC = acetic acid

Table 4: contiuous dialysis fermentations - results

Experiment		1	2	3	4	5	6	7	8	9	10
Temperature	[°C]	37	70	90	90	90	90	90	90	90	100
Mass transport											
Volumetric loading	[g_{TS}/(Ld)]	2.0	2.8	2.8	4.7	7.4	13.1	4.9	5.0	5.4	2.0
Retention time	[d]	7.6	8.1	11.9	1.4	1.5	1.2	5.6	5.8	4.0	10.6
Membrane flow	[mL/d]	102	115	174	-355	128	-312	328	384	120	11
Spec. Permeability	[d^{-1}]	0.613	1.794	3.174	2.474	2.306	10.353	0.164	0.234	0.332	1.587
Carbon balance											
Feed											
solid	[g_C/d]	0.914	1.200	1.200	1.980	3.143	5.544	6.885	6.998	7.646	0.850
liquid *	[g_C/d]	0.286	0.452	0.503	0.829	1.317	2.323	2.884	2.932	3.203	0.374
Effluent											
solid	[g_C/d]	0.335	0.389	0.299	1.328	1.717	3.318	5.192	4.849	5.236	0.172
liquid	[g_C/d]	0.054	0.068	0.037	0.390	0.642	0.887	1.624	1.409	1.662	0.041
- VFA	[g_C/d]	0.006	0.007	0.004	0.056	0.072	0.115	0.156	0.159	0.176	0.000
Dialysate											
liquid	[g_C/d]	0.250	0.798	0.957	0.697	1.392	2.223	2.002	2.420	2.235	0.619
- VFA	[g_C/d]	0.144	0.169	0.196	0.143	0.265	0.160	0.391	0.573	0.254	0.049
Gas production	[g_C/d]	0.562	0.397	0.409	0.393	0.709	1.440	0.951	1.252	1.717	0.392
Solid matter degradation		**63%**	**68%**	**72%**	**33%**	**45%**	**40%**	**24%**	**30%**	**31%**	**80%**

* Spontaneous solubilization at 37 °C = 23%, at 70 °C = 27%, at 90 °C and 100 °C = 30%

gas production (CO_2) calculated from carbon balance. No methane formation was observed at 90 °C and 100 °C.

The experiments were evaluated by their carbon balances. Figure 17 shows the carbon balance for all 10 experiments.

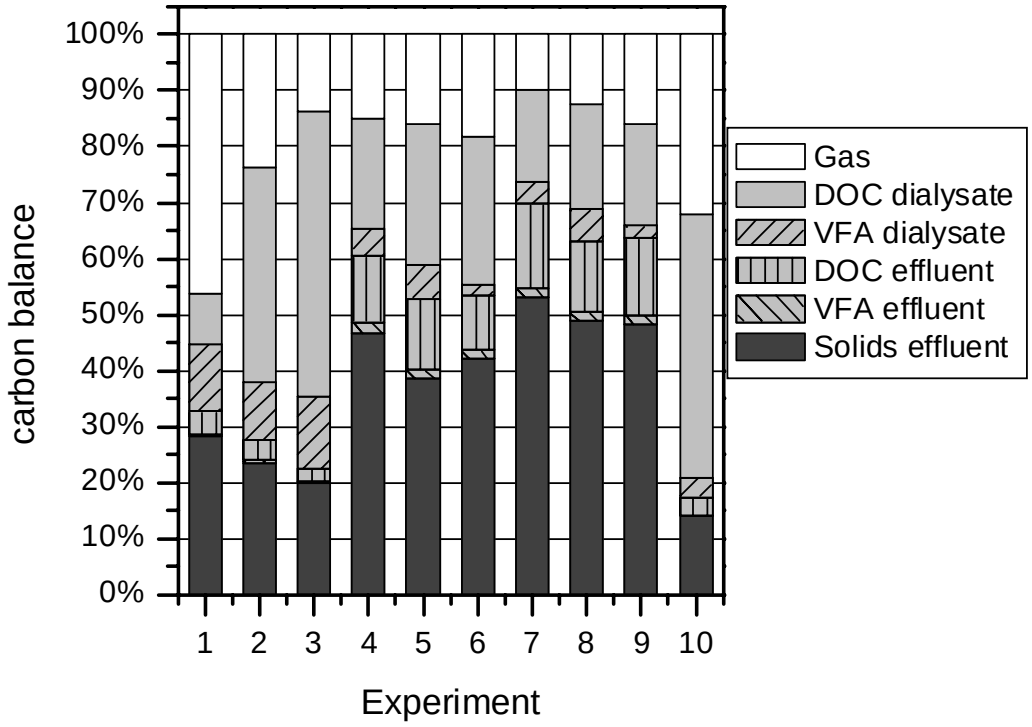

Figure 17 Comparison of 10 experiments conducted in the continuous dialysis reactor. Gas production (CO_2) was calculated from the gap in the carbon balance.

3.2 Impact of temperature

At first it becomes apparent that the solid matter degradation performance increases with temperature. Figure 18 compares four experiments (1-3, 10) with similar experimental conditions, except for pH in experiment 10. The experiment at 100 °C differs in pH and dialysate exchange rate from the other 3. Comparing the experiments at 37, 70 and 90°C, an increase in liquefaction and a decrease of gasification can be observed with increasing temperature.

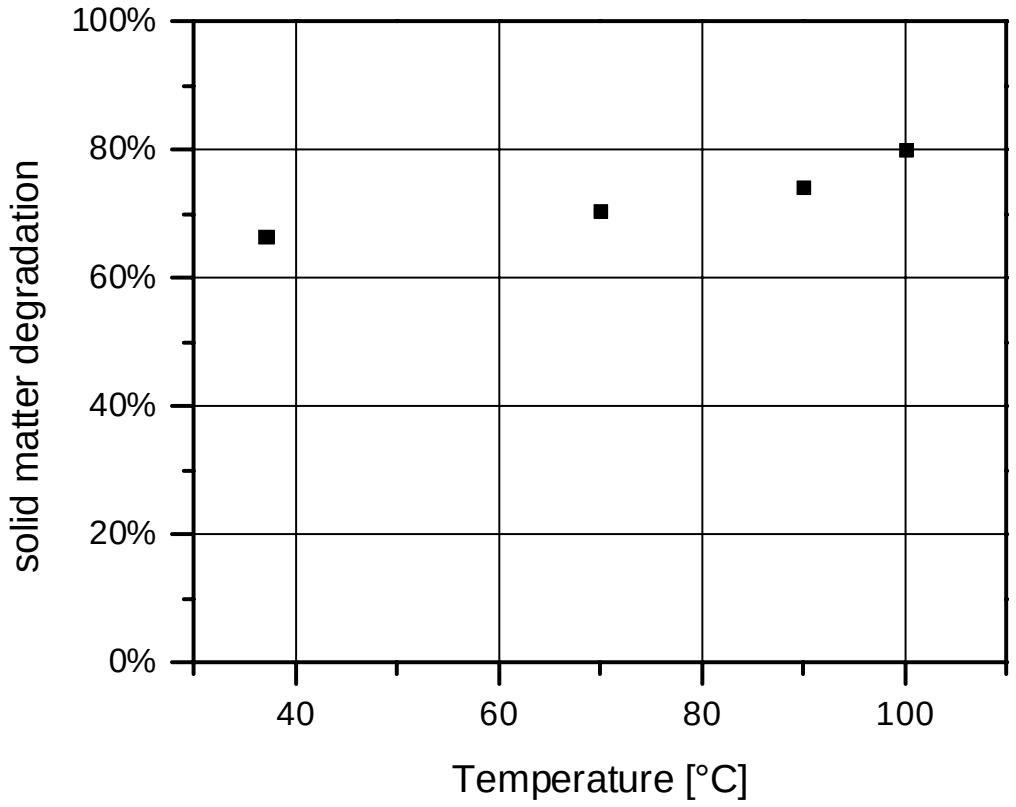

Figure 18: solid matter degradation in a continuous dialysis reactor at different temperatures. During all experiments the volumetric loading rate was 2 g/(L d).

The highest degradation performance of 80% was visible at 100°C (experiment 10). The experiment is shown in detail in figure 19. In the first days lot of carbon was flushed from the fermentor. More carbon left the fermentor than was fed to the fermentor in this time. After three days a steady state was achieved, the in- and outgoing carbon streams remained more or less constant throughout the experiment.

3.3 Mathematical model

The comparison of the 10 experiments often led to the problem, that not all experimental parameters were constant whereas only one parameter was changed, to monitor the influence of this special parameter on the liquefaction performance. Most of the changes were on purpose, some changes – especially the adjustment of the retention time and trans-membrane flow – were the effects of slight changes in the fermentor set up and could not be influenced.

To achieve a profound understanding of the system behavior a mathematical model was developed. Balances for solid carbon in the cultivation chamber and dissolved organic carbon in the cultivation chamber and dialysate chamber were drawn. The model assumptions are:

- Steady state, no accumulation in the fermentor
- Solids are liquefied and converted to DOC
- Gas is produced form the liquid substrate (DOC)
- DOC can pass the membrane with convective flow

The model and its equations are shown in figure 20. For the nomenclature all indexes for the cultivation chamber are 1, the index for the dialysis chamber is 2.

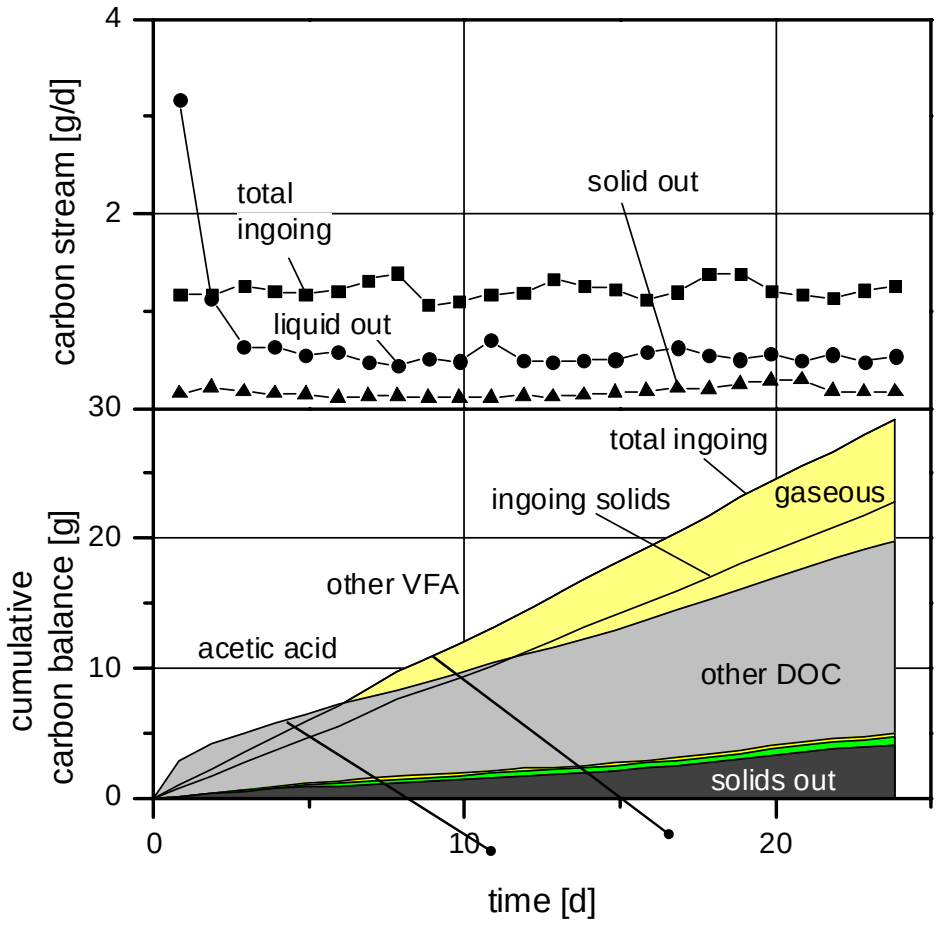

Figure 19: Carbon balance of the continuous dialysis fermentation of a hyperthermophilc consortium at 99.5 °C and pH 8. The retention time in the cultivation chamber was 10.5 d, the hydraulic retention time in the dialysate chamber was 0.25 d. 2% ESA-Substrate was used. The upper plot shows the ingoing and outgoing carbon streams, the lower plot displays the cumulative carbon balance. At the end of the experiment 29.3g_C (100%) entered the fermentor. 15.7 g_C (54%) were liquefied, 1.1 g_C (4%) VFA (C2-C7) were produced. 4.1 gC left the fermentor in solid form. The gap in the balance was 8.4 gC (32%) and corresponds to the gas production (not measured). The solid matter degradation was 80%.

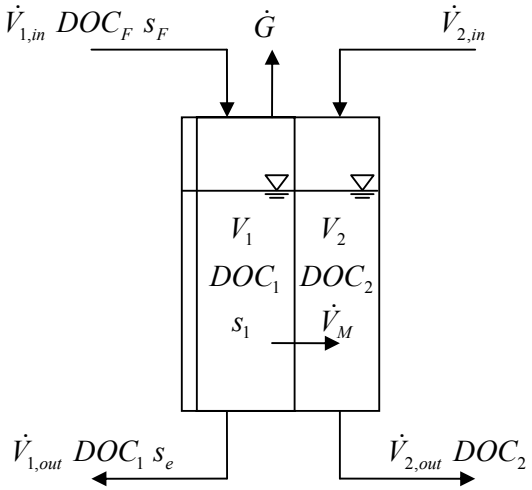

Figure 20: Balance model of the continuous dialysis reactor

Legends can be found in Appendix

Equation 1 deals with the solid matter in the cultivation chamber.

$$\frac{ds_1}{dt} = \frac{\dot{V}_{1,in}}{V_1} \cdot s_F - \frac{\dot{V}_{1,out}}{V_1} \cdot s_E - \dot{r} \qquad (1)$$

$\dot{r}$ describes the rate for the microbial liquefaction of solids. For the balance of DOC in the cultivation and dialysis chamber two different cases must be distinguished. In case one the net flow of media is from the cultivation chamber to the dialysate chamber. The DOC concentrations are described by equations 2a and 3a. In case two the net flow is from the dialysate chamber to the cultivation chamber. In this case the DOC concentrations are described by equation 2b and 3b.

$\dot{V}_M \geq 0$

$$\frac{dDOC_1}{dt} = \frac{\dot{V}_{1,in}}{V_1} \cdot DOC_F - \frac{\dot{V}_{1,out}}{V_1} \cdot DOC_1 + \dot{r} - \dot{g} - \frac{PA}{V_1} \cdot (DOC_1 - DOC_2) - \frac{\dot{V}_M}{V_1} \cdot DOC_1 \qquad (2a)$$

$$\frac{dDOC_2}{dt} = \frac{PA}{V_2} \cdot (DOC_1 - DOC_2) - \frac{\dot{V}_{2,out}}{V_2} \cdot DOC_2 + \frac{\dot{V}_M}{V_2} \cdot DOC_1 \qquad (3a)$$

$\dot{V}_M < 0$

$$\frac{dDOC_1}{dt} = \frac{\dot{V}_{1,in}}{V_1} \cdot DOC_F - \frac{\dot{V}_{1,out}}{V_1} \cdot DOC_1 + \dot{r} - \dot{g} - \frac{PA}{V_1} \cdot (DOC_1 - DOC_2) - \frac{\dot{V}_M}{V_1} \cdot DOC_1 \qquad (2b)$$

$$\frac{dDOC_2}{dt} = \frac{PA}{V_2} \cdot (DOC_1 - DOC_2) - \frac{\dot{V}_{2,out}}{V_2} \cdot DOC_2 + \frac{\dot{V}_M}{V_2} \cdot DOC_2 \qquad (3b)$$

The membrane flow is defined from the inner chamber to the outer chamber in equation 4

$$\dot{V}_M = \dot{V}_{1,in} - \dot{V}_{1,out} \qquad (4)$$

The solid matter degradation term $\dot{r}$ is dependent from the volumetric loading rate B_V, or more precisely from the solid carbon loading rate $B_{TS,C}$ [g_C/(L d)] as depicted in figure 21. The maximal solid matter degradation term is equal to the solid carbon loading rate; this is equivalent to 100% degradation.

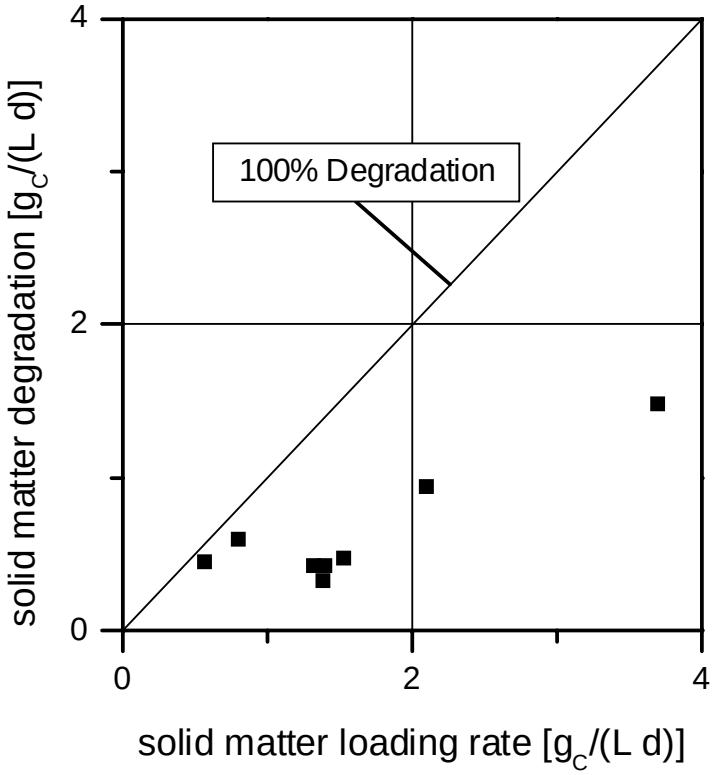

Figure 21: solid matter degradation rate $\dot{r}$ versus solid carbon loading rate $B_{TS,C}$ for the hyperthermophilic dialysis culture. The 45° line indicates the maximal solid matter degradation at given carbon loading rate.

The exact relationship between the solid carbon loading rate and the solid carbon degradation rate is given in equation 5. It depends on the amount of dissolved organic carbon in the cultivation chamber. The higher the DOC concentration in the cultivation chamber the lower is the solid carbon degradation term.

$$\dot{r} = B_{TS,C} \cdot \frac{K_I}{K_I + DOC_1} \qquad (5)$$

This model behavior can be explained by the nutritional demands of the microorganisms. When a high liquid substrate concentration is present, the microorganisms do not have to

secrete carbohydrate degrading enzymes. Instead they can use the liquefied substrate for growth and maintenance.
Equation 5 has its limits, since it cannot be used to calculate a maximal degradation rate. Therefore it is just valid in the measured boundaries 0.5 $g_C/(L\ d) < B_{TS,C} < 4\ g_C/(L\ d)$ and 0.2 $g_C/L < DOC_1 < 2\ g_C/L$.
Figure 22 visualizes equation 5. Fitting the calculated curve to the measured points an inhibition constant of $K_I = 0.55 g_C/L$ is obtained. Here the degradation efficiency is plotted against DOC_1. The degradation efficiency is defined by equation 6 as the solid carbon degradation rate divided by the solid carbon loading rate.

$$\eta = \frac{\dot{r}}{B_{TS,C}} \qquad (6)$$

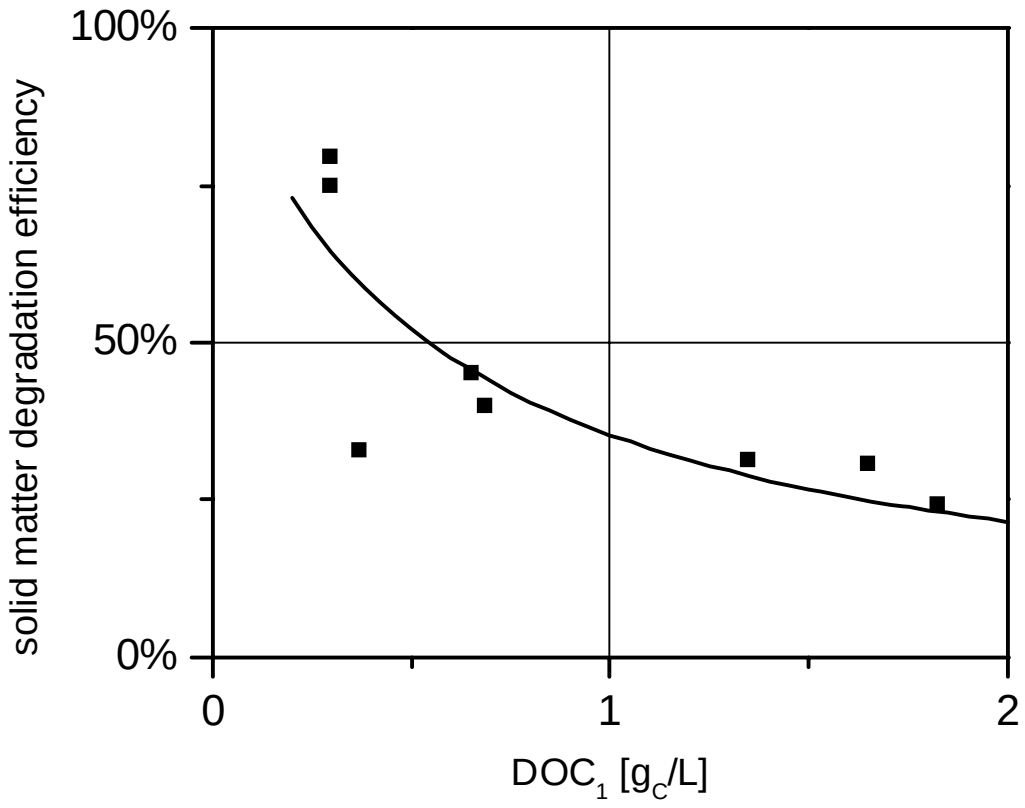

Figure 22: solid carbon degradation efficiency versus dissolved organic carbon in culture chamber for the hyperthermophilic culture. From the measured values the inhibition constant is fitted to $K_I = 0.55 g_C/(L\ d)$. Except for experiment 4 all efficiencies lay within 10% of the calculated curve.

The next parameter which has to be described is the gas production rate $\dot{g}$. It is proportional to the microbial solid carbon degradation rate. This was suspected, because the gas production is

a good indicator for microbial activity. The exact relationship is shown in figure 23. Its mathematical formulation is stated in equation 7.

$$\dot{g} = K_G \cdot \dot{r} \quad (7)$$

The value for K_G is 0.55.

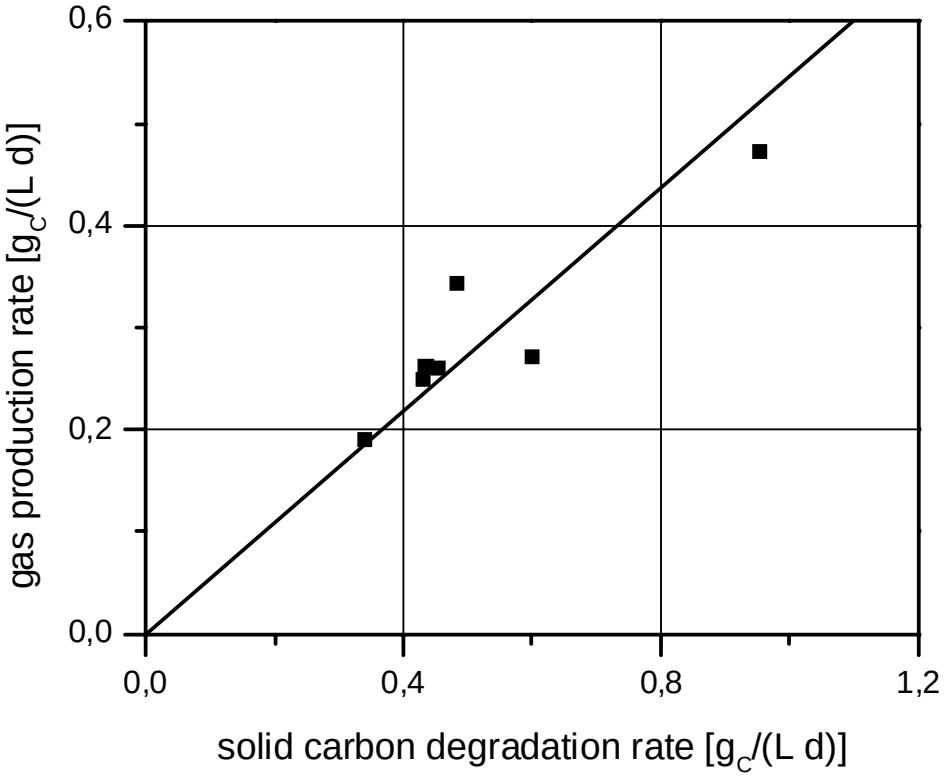

Figure 23: gas production rate versus solid carbon degradation rate. The shown data points cover all experiments (3-10) with the hyperthermophilic consortium at 90 °C and 100 °C. The dimensionless gas production constant K_G is determined to 0.55.

The last parameter which is needed for the description of the system is the permeability of the product DOC. It can be determined from equation 3a or 3b, if the active membrane area is known. The product of permeability and membrane area, PA, is equal to the mass transport coefficient known from literature about heat and mass transfer. PA divided by the cultivation volume is the specific mass transport coefficient. It is listed in figure 24. The permeability is a function of the molar weight distribution of the DOC. A high permeability equals a low average molar weight of the DOC. Comparing experiment 1-3 and 10, all done at the same volumetric loading rates in a dialysis membrane reactor, it becomes visible that the permeability increases with temperature from 37 °C to 90 °C and then decreases again. This indicates that degradation at 90 °C yields the smallest molecules and 37 °C the biggest. Experiment 6 resulted in a very high permeability of DOC. The permeability here is about 4

times higher than the average permeability at 90 °C in the dialysis membrane reactor. A reason for this was not found, a measurement or calculation error can be excluded. Probably the biomass was very active and produced a greater number of small sized molecules. An unusual high production of acetic acid or VFA was not observed in experiment 6.
The experiments 7-9 were carried out with the submerged membrane module system. Here, the specific mass transport coefficient was approximately ten times lower than in experiment 3-5. The increase of dialysate flow rate from experiment 8 to experiment 9 by a factor of three lead to an increase of permeability by 40%, however the specific mass transport coefficient was still one order of magnitude lower than in the dialysis membrane reactor, so the submersed membrane approach was cancelled.

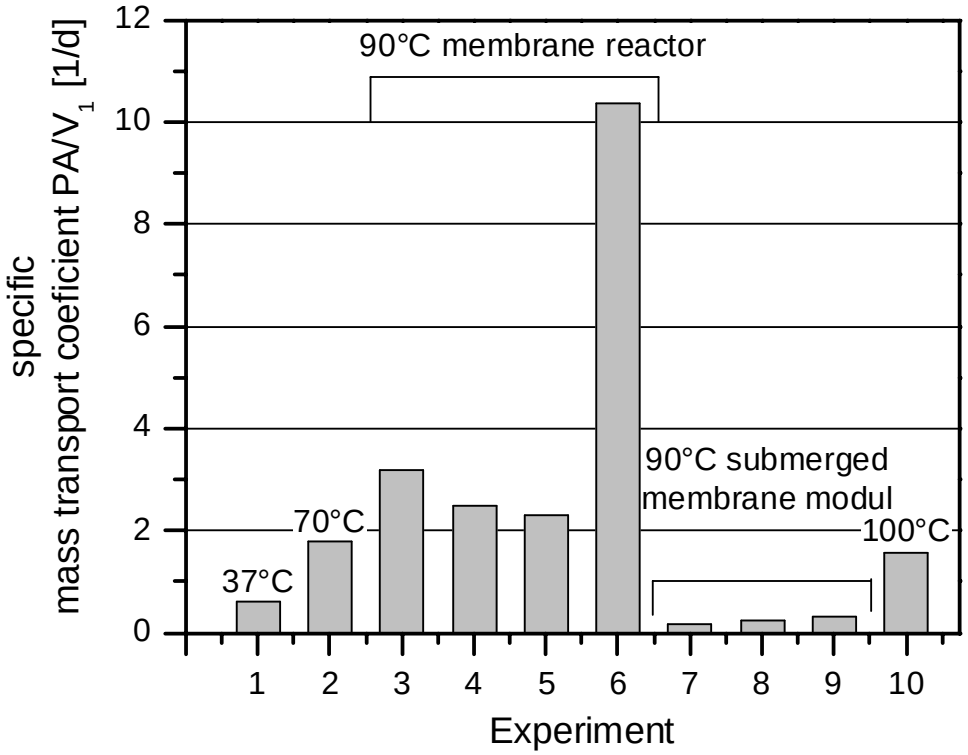

Figure 24: specific mass transport coefficient of all experiments, determined by a carbon balance of the dialysate, the experiments at 37 °C, 70 °C, and 100 °C were conducted in a dialysis membrane reactor. Form the experiments 3-6, all carried out at the same temperature and with the same membrane set up, the last experiment (6) shows a four times higher specific mass transport coefficient, than the rest. Remarkable is also the comparatively lower specific mass transport coefficient in the experiments with a submersed membrane module, despite of the higher membrane area.

For modeling purposes the permeability was set to 0.5×10^{-2} cm/min. This is the average value of the permeability of experiment 3, 4, and 5.

With these parameters the model was solved for stationary conditions. A comparison between the measured and the calculated concentrations of solid in the effluent s_E, and the dissolved organic carbon in the effluent (DOC_1) and dialysate (DOC_2) is shown in figure 25.

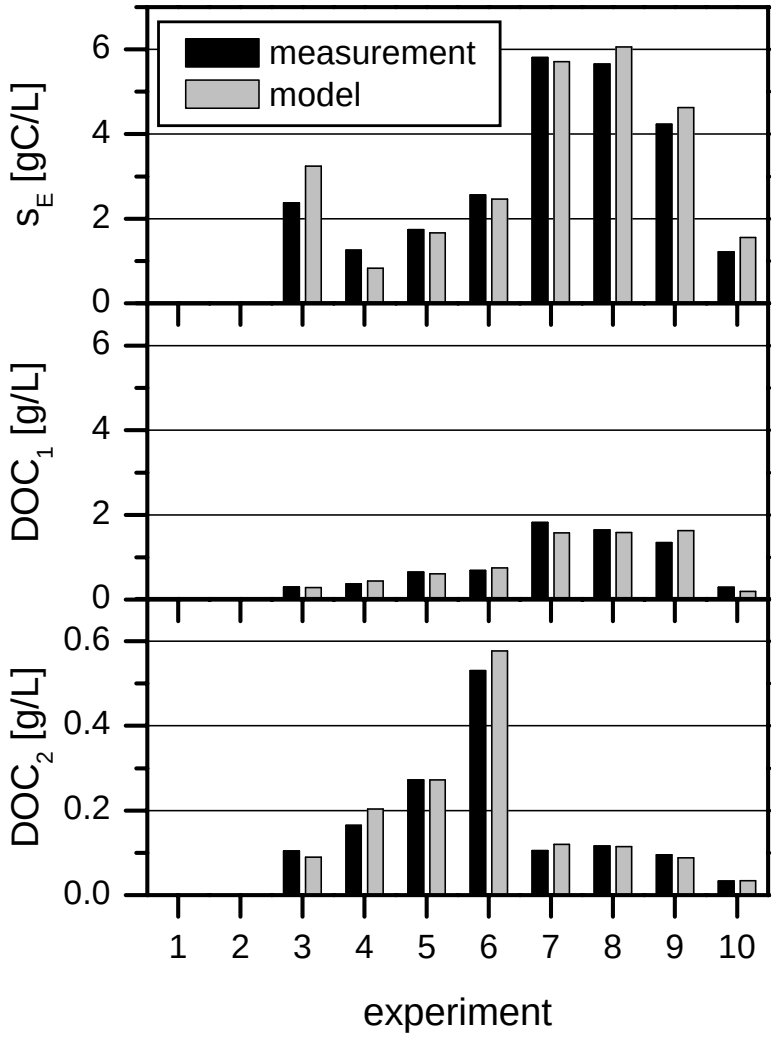

Figure 25: Comparison between the measured and the modeled concentrations. Solids in the effluent (s_E), dissolved organic carbon in the effluent (DOC_1) and dialysate (DOC_2) were modeled with good accuracy.
The model parameters were determined in figure 21, 22, and 23 for hyperthermophilic conditions.

With the modeled concentrations the modeled carbon balance can be calculated. It is shown in figure 25.

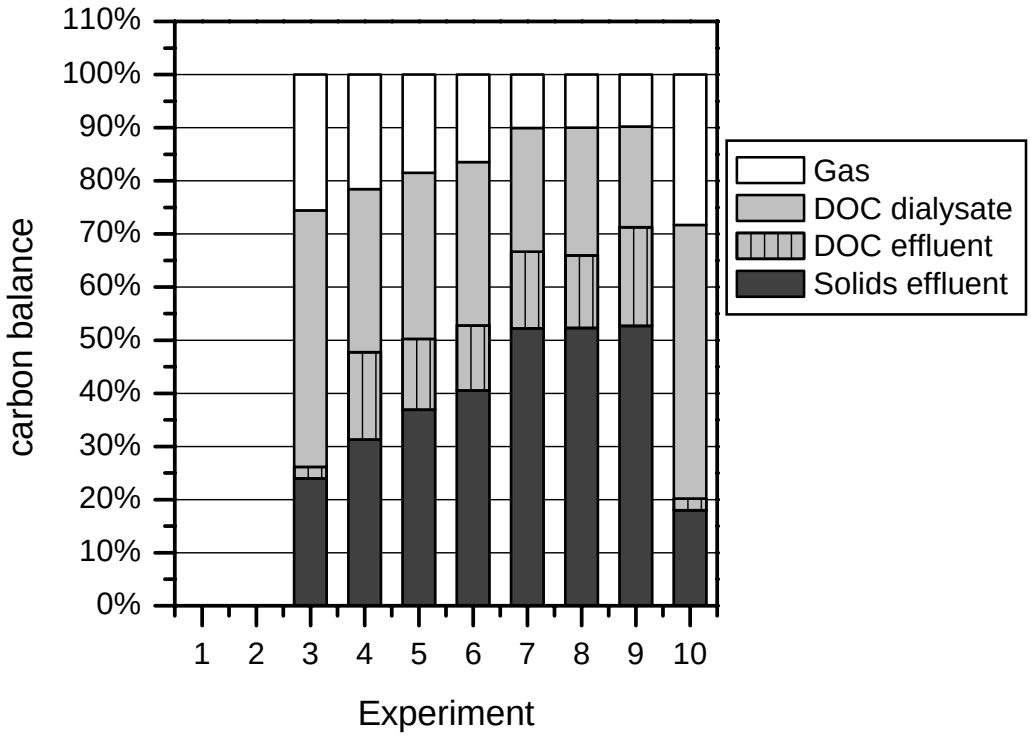

Figure 26: Modeled carbon balances for the experiments at hyperthermophilic conditions. Solid carbon in the effluent, and dissolved organic carbon in the effluent and dialysate were modeled. Input parameters were volume flows, reactor volume, and solid content in the feed. Model parameters were the specific mass transport coefficient (PA/V_1), the inhibition constant K_I, and the gas production coefficient K_G.

Except for experiment 4 all calculated carbon balances were verified by the measured carbon balances.

3.4 Composition of the fermentor effluent and dialysate

The composition of the dialysate and fermentor effluent was determined in experiment 6.

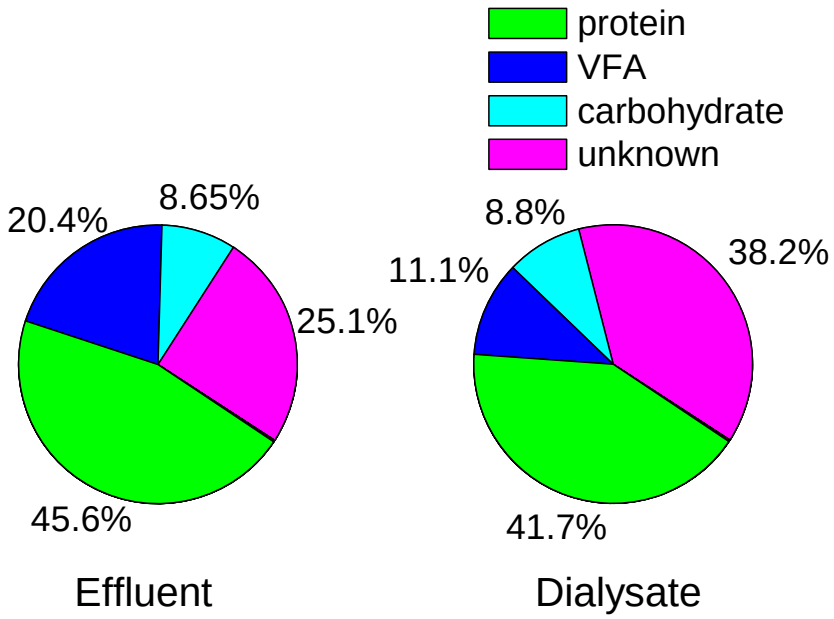

Figure 27: composition of the effluent and dialysate. Samples were taken from the effluent / dialysate of one day at the end of the fermentation. Free amino acids and free sugars are not shown, since their concentration is below 1%

Both dialysate and effluent have similar concentration patterns. The largest group of soluble molecules is the protein fraction (46% effluent, 42% dialysate). The high concentration of protein in the dialysate is remarkable, because normally large protein do not diffuse through the membrane. However, throughout the fermentation often a convective stream over the membrane was monitored. The cut off of the membrane in ultra filtration mode is 300 kDa, so most of the proteins can be pressed through the membrane.
The second largest fraction is still unknown (25% effluent, 39% dialysate). Possible molecules are alcohols and aldehydes or non volatile fatty acids, like pyruvic or succinic acid.
The next fraction is the volatile fatty acid fraction (20% effluent, 11% dialysate), which consists mainly of acetic acid. Higher VFA (C3-C7) are just found in traces. Carbohydrates are found in both streams at the same concentration (9%). Free amino acids are just found in very small concentrations (0.2%). The same is valid for free sugars (0.02%).
Figure 27 gives a graphical overview over the found substances.
Besides quantitative determination a qualitative GC-MS analysis was done. The analysis revealed traces of alkanes and alcylamides in dialysate and effluent. These molecules or their precursors are natural degradation products of plant fibers (Himmel et al., 1994) Alcoholes, and fatty acids can be reduced enzymatically by Alcoholdehydrogenases (ADH) and

Alcoholoxidoreductases (AOR) to alkanes. Microbial alkylamide formation was monitored in sewage sludge reactors (Thomas, 1981).
In the dialysate also a peak was found, indicating the presence of a terpene. Terpenes are C_5-bodies, known as aromatic oils, but are neither oils nor aromatics.
No halogenated molecules were found in both streams. Halogens show a very characteristic isotropic pattern in the MS; this pattern was not found. Aromatic molecules or arylgroups were not found as well.

4 OUTLOOK:

The hyperthermophilic liquefaction unit showed good results in the liquefaction of lignocellulose. As by-product vast amounts of dialysate are produced, which have to be recycled. Aerobic processes, commonly used for this purpose were out of question, because of the high sludge production and oxygen demand of this process step.
Anaerobic processes have the great benefit of a low sludge production, but are normally applied for higher substrate concentrations. To achieve high loading rates at low substrate concentrations very low retention times are necessary. Because of the slow growth of methanogenic bacteria and archaea with common doubling times of 100 h an efficient biomass retention system must be applied. Such systems are: membrane cell recycle, sedimentation of active biomass and biomass fixation. All systems have their advantages and disadvantages. Membrane cell recycle systems have a high efficiency and an almost sterile filtrate but membrane performance decreases significantly and the membrane will plug after a while. Sedimentation systems are stable, but need huge sedimentation vessels and often only display moderate efficiencies. The fixed bed reactor is easily scaled up; it is very stable at varying loading rates. As disadvantage must be mentioned, that the bio-film on the carriers tends to grow over time. This behavior leads to the plugging of the fermentor and to the formation of short-cuts through the packing of the fermentor.

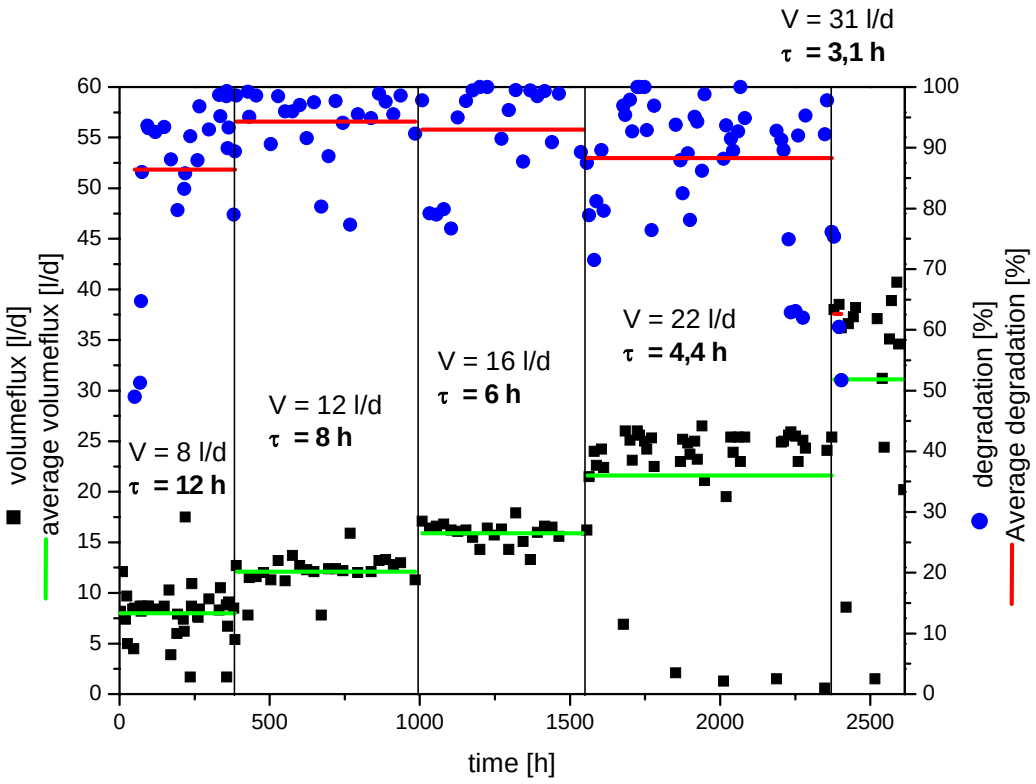

Figure 28 Degradation of artificial waste water (dialysate from the hyperthermophilic liquefaction unit) with 5 mM acetic acid as sole carbon source at low volumetric loading rates in a mesophilic (37 °C) anaerobic radial flow fixed bed reactor.

Despite of this disadvantage we decided to use the latter system for the anaerobic purification of the loaded dialysate. The carbon concentrations in the dialysate ranged from 0.05 g/L to 0.6 g/L. Thus an artificial dialysate stream containing 5 mM – 20 mM acetic acid (0.12 g_C/L – 0.48 g_C/L) was composed and fed to the fixed bed reactor. The experiment with 5 mM acetic acid is shown in figure 28. Hydraulic retention times of 12 h, 8 h, 6 h, 4.4 h, and 3.1 h were applied. The experiment was repeated with 10 mM and 20 mM (data not shown). The degradation performance at different retention times is shown in figure 29. Retention times higher than 6 h yielded almost complete degradation (>98%) of the acetic acid. The degradation performance was independent from the substrate concentration. The highest loading rate achieved with this set up was 2.9 g_C/(L d) with a degradation efficiency of 80%. These loading rates are comparable of the loading rates of the hyperthermophilic reactor. Hence a second apparatus comparable in size to the hyperthermophilic reactor is required. Higher loading rates and therefore smaller reactors require a concentration of the substrate.

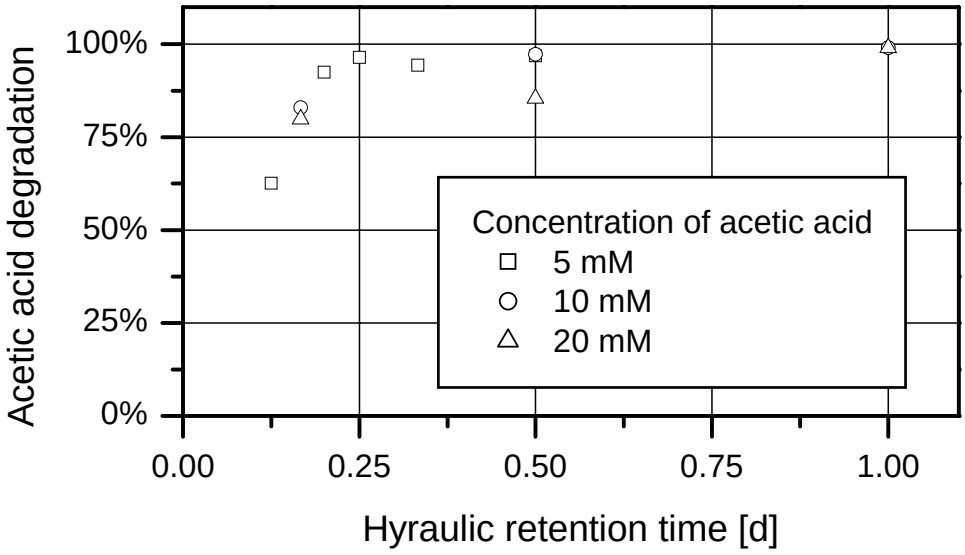

Figure 29: Degradation performance of an anaerobic mesophilic radial flow fixed bed reactor for diluted waste waters. At retention times smaller than 6 h the degradation performance decreases. The degradation performance is independent from the substrate concentration.

A better way to degrade diluted waste waters is the use of phototrophic micro-organisms as suggested in the MELiSSA Loop. Photoheterotrophic organisms can grow on a combination of CO_2 plus light and organic carbon sources. Their kinetics do not entirely depend on the concentration of the organic substrate. Photohereothrophic growth and substrate concentration are decoupled.

5 CONCLUSIONS:

In the last five years we were able to isolate a hyperthermophilic consortium capable to degrade solid substrate containing lignocellulose. First trials in batch and continuous mode were done showing just a poor conversion of about 40%. At the end of phase one dialysis fermentations were introduced.

Phase two began with experiments concerning the use of loaded dialysate as substrate for a methanogenic fixed bed reactor. If was demonstrated, that diluted wastewater streams could be purified in this reactor with conversions of up to 99.5%. In parallel micro-organisms from literature (partly isolated form partner 5, DTU) were tested for growth in a bioreactor, using cellulose and cellobiose as carbon source. The influence of stirring on growth and product formation was depicted, a low stirrer speed and no gassing is recommended for the fermentation of these microorganisms. Dialysis fermentations in the dialysis membrane reactor and with external dialysis membranes were done, leading to higher biomass yields than the batch reactor.

The continuous dialysis fermentation was tested for the extreme- (70 °C) and hyperthermophilic (90 °C) microorganisms, a mesophilic reactor was run at well. Comparison of these three experiments showed the greatest liquefaction potential at hyperthermophilic conditions. Further experiments at 90 °C were conducted at higher loading rates. Higher loading rates were achieved by higher substrate concentrations and lower retention times.

The hyperthermophilic consortium was grown in solid free media. Surprisingly, highest biomass concentration was found at pH8 and 100 °C. Therefore, an experiment at this condition was run and yielded the highest degradation performance of 80%.

Submerged membrane modules were tested. This modules offer a much greater membrane area compared to the dialysis membrane reactor. However, the mass transport over this membrane was very low. The main mass transport resistance was the bad mixing outside of the membrane module.

At the end, the system behavior was modeled on the basis of 8 experiments at hyperthermophilic conditions. The model describes the system behavior quite well. Analysis of the model suggests a high membrane area and high dialysate stream for better performance and higher conversion rates.

This can only be achieved by the use of external membrane modules. These modules are on the market, but their use is not recommended at elevated temperatures for longer times. In general, the membrane material can stand temperatures up to 130 °C, but the housing, the glue and the fittings can not. A design of a thermostable dialysis membrane module is therefore possible. Using such membrane modules require a solid free inlet stream. Rotating filter meshes offer a good separation performance at reduced fouling potential. Such systems are on the market, offered by the company Bioengineering, CH in liter to cubic meter scale. A combination of these new technologies promises to maintain the high conversion rates even at large volumetric loading rates.

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APPENDIX A´LEGEND

Volume- and carbon flows

Volumeflows	name	Unit
Feed	$\square_{1,in}$	[L/d]
fresh dialysate	$\square_{2,in}$	[L/d]
Efluent	$\square_{1,out}$	[L/d]
loaded dialysate	$\square_{2,out}$	[L/d]

Volume

Inner chamber / reaction volume	V_1	[L]
Outer chamber	V_2	[L]

Carbon flow, Amount of carbon

Mass flow, gaseous phase	$\dot{G}$	[g_C/d]
Accumulation	ϕ	[g_C]

Solid matter concentration

Feed, initial weight solids	TS_F	[g_{TS}/L]

Concentration of carbon

Feed, concentration of carbon n solid matter	TC_F	[g_C/g_{TS}]
Feed, measured solid carbon at reactor-temperature	s_F	[g_C/L]
Feed, dissolved carbon at reactor-temperature	DOC_F	[g_C/L]
fresh Dialysate	zero	[g_C/L]
Reactor, solid	s_1	[g_C/L]
Reactor, liquid	DOC_1	[g_C/L]
effluent, solid	s_E	[g_C/L]
effluent, liquid	DOC_1	[g_C/L]
loaded dialysate, liquid	DOC_2	[g_C/L]
Dialysis-chamber, liquid	DOC_2	[g_C/L]